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PROVISIONAL APPLICATION COVER SHEET

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TITLE OF THE INVENTION (280 characters max)

STABILIZED VIRAL ENVELOPE PROTEINS AND USES THEREOF

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STABILIZED VIRAL ENVELOPE PROTEINS AND USES THEREOF

The invention disclosed herein was made with Government support under NIH Grant Nos. R01 AI39420, R01 AI42382, R01 AI45463, R21 AI44291, R21 AI49566, and U01 AI49764 from the Department of Health and Human Services. Accordingly, the government has certain rights in this invention.

Throughout this application, various publications are referenced. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the art to which this invention pertains.

Background of the Invention

The human immunodeficiency virus (HIV) is the agent that causes AIDS, a lethal disease characterized by deterioration of the immune system. The initial phase of the HIV replicative cycle involves the attachment of the virus to susceptible host cells followed by fusion of viral and cellular membranes. These events are mediated by the exterior viral envelope glycoproteins, which are first synthesized as a fusion-incompetent precursor envelope glycoprotein (env) known as gp160. The gp160 glycoprotein is endoproteolytically processed to the mature envelope glycoproteins gp120 and gp41, which are noncovalently associated on the surface of the virus. The gp120 surface protein contains the high affinity binding site for human CD4, the primary receptor for HIV, as well as domains that interact with fusion coreceptors, such as the chemokine receptors CCR5 and CXCR4. The gp41 protein spans the viral membrane and contains at its amino-terminus a sequence of amino acids important for the fusion of viral and cellular membranes. The HIV envelope glycoproteins assemble as noncovalent oligomers, almost certainly trimers, of gp120/gp41 on the virus surface. The detailed events of viral

entry remain poorly understood but involve gp120 binding first CD4 then a fusogenic chemokine receptor, followed by gp41-mediated virus-cell fusion.

Because of their location on the virion surface and central role in mediating viral entry, the HIV envelope glycoproteins provide important targets for HIV vaccine development. Although most HIV-infected individuals mount a robust antibody (Ab) response to the envelope glycoproteins, most anti-gp120 and anti-gp41 Abs produced during natural infection bind weakly or not at all to virions and are thus functionally ineffective. These Abs are probably elicited and affinity matured against "viral debris" comprising gp120 monomers or improperly processed oligomers released from virions or infected cells. (Burton and Montefiori, AIDS, 11 [Suppl A]: 587, 1997)

Several preventive HIV-1 subunit vaccines have been tested in Phase I and II clinical trials and a multivalent formulation is entering Phase III testing. These vaccines have contained either monomeric gp120 or unprocessed gp160 proteins. In addition, the vaccines mostly have been derived from viruses adapted to grow to high levels in immortalized T cell lines (TCLA viruses). These vaccines have consistently elicited Abs which neutralize the homologous strain of virus and some additional TCLA viruses. However, the Abs do not potently neutralize primary HIV-1 isolates (Mascola *et al.*, J. Infec. Dis. 173:340, 1996). Compared with TCLA strains, the more clinically relevant primary isolates typically possess a different cellular tropism, show a different pattern of coreceptor usage, and have reduced sensitivity to neutralization by soluble CD4 and Abs. These differences primarily map to the viral envelope glycoproteins (Moore and Ho, AIDS, 9 [Suppl A]:S117-S136, 1995).

<u>The importance of oligomerization in envelope glycoprotein structure</u>

There is a growing awareness that current-generation HIV subunit vaccines do not adequately present key neutralization epitopes as they appear on virions (Parren et al, Nat. Med. 3:366, 1997). There are several ways in which the native structure of virions affects the presentation of antibody epitopes. Firstly, much of the surface area of gp120 and gp41 is occluded by inter-subunit interactions within the trimer. Hence several regions of gp120, especially around the N- and C-termini, that are well exposed (and highly immunogenic) on the monomeric form of the protein, are completely inaccessible on the native trimer (Moore et al, J. Virol 68:469, 1994). This means that a subset of Abs raised to gp120 monomers are irrelevant, whether they arise during natural infection (because of the shedding of gp120 monomers from virions or infected cells) or after gp120 subunit vaccination. This provides yet another level of protection for the virus; the immune system is decoyed into making Abs to shed gp120 that are poorly reactive, and hence ineffective, with virions.

A second, more subtle problem is that the structure of key gp120 epitopes can be affected by oligomerization. A classic example is provided by the epitope for the broadly neutralizing human MAb IgG1b12 (Burton et al. Science 266:1024, 1994). This epitope overlaps the CD4-binding site on gp120 and is present on monomeric gp120. However, IgG1b12 reacts far better with native, oligomeric gp120 than might be predicted from its monomer reactivity, which accounts for its unusually potent neutralization activity (77,99-103). Thus the IgG1b12 epitope is oligomer-dependent, but not oligomer-specific. The converse situation is more common, unfortunately; many Abs that are strongly reactive with CD4-binding site-related epitopes on monomeric gp120, fail to react with the native trimer, and consequently do not neutralize the virus. In some undefined way, oligomerization of gp120 adversely affects the structures recognized by these Mabs. (Fouts et al., J Virol 71: 2779, 1997).

A third example of the problems caused by the native structure of the HIV-1 envelope glycoproteins is provided by gp41 MAbs. Only a single gp41 MAb (2F5) is known to have strong neutralizing activity against primary viruses (Trkola et al., J Virol, 69: 6609, 1995), and among those tested, 2F5 alone is thought to recognize an intact, gp120-gp41 complex (Sattentau et al, Virology 206: 713, 1995). All other gp41 MAbs that bind to virions or virus-infected cells probably react with fusion-incompetent gp41 structures from which gp120 has dissociated. Since the most stable form of gp41 is this post-fusion configuration (Weissenhorm et al, Nature, 387: 426, 1997), it can be supposed that most anti-gp41 Abs are raised (during natural infection or after gp160 vaccination) to an irrelevant gp41 structure that is not present on the pre-fusion form.

Despite these protective mechanisms, most HIV-1 isolates are potently neutralized by a limited subset of broadly reactive human monoclonal antibodies (MAbs), so induction of a relevant humoral immune response is not impossible. Mab IgG1b12, blocks gp120-CD4 binding; a second (2G12; Trkola et al. J Virol 70: 1100, 1996) acts mostly by steric hindrance of virus-cell attachment; and 2F5 acts by directly compromising the fusion reaction itself. Critical to understanding the neutralization capacity of these MAbs is the recognition that they react preferentially with the fusion-competent, oligomeric forms of the envelope glycoproteins, as found on the surfaces of virions and virus-infected cells. (Parren et al J. Virol 72: 3512, 1998). This distinguishes them from their less active peers. The limited number of MAbs that are oligomer-reactive explains why so few can neutralize primary viruses. Thus with rare exceptions, neutralizing anti-HIV Abs are capable of binding infectious virus while non-neutralizing Abs are not (Fouts et al AIDS Res Human Retrovir. 14: 591, 1998). Neutralizing Abs also have the potential to clear infectious virus through effector functions, such as complement-mediated virolysis.

Modifying the antigenic structure of the HIV envelope glycoproteins

HIV-1 has evolved sophisticated mechanisms to shield key neutralization sites from the humoral immune response, and in principle these mechanisms can be "disabled" in a vaccine. One example is the V3 loop, which for TCLA viruses in particular is an immunodominant epitope that directs the antibody response away from more broadly conserved neutralization epitopes. HIV-1 is also protected from humoral immunity by the extensive glycosylation of gp120. When glycosylation sites were deleted from the V1/V2 loops of SIV gp120, not only was a neutralization-sensitive virus created, but the immunogenicity of the mutant virus was increased so that a better immune response was raised to the wild-type virus (Reitter et al, Nat Med 4:679, 1998). Similarly, removing the V1/V2 loops from HIV-1 gp120 renders the conserved regions underneath more vulnerable to Abs (Cao et al, J. Virol. 71: 9808, 1997), although it is not yet known whether this will translate into improved immunogenicity.

Of note is that the deletion of the V1, V2 and V3 loops of the envelope glycoproteins of a TCLA virus did not improve the induction of neutralizing Abs in the context of a DNA vaccine (Lu et al, AIDS Res Human Retrovir 14:151, 1998). However, the instability of the gp120-gp41 interaction, perhaps exacerbated by variable loop deletions, may have influenced the outcome of this experiment. DNA plasmid, viral vector and other nucleic acid-based HIV vaccines may thus benefit from the gp120-gp41 stabilizations described in this invention. By increasing the time that the gp120-gp41 complex is presented to the immune system, stabilized envelope proteins expressed in vivo provide a means in principle to significantly improve upon the immune response elicited during natural infection.

Native and non-native oligomeric forms of the HIV envelope

glycoproteins

Current data suggest that on the HIV virion three gp120 moieties are non-covalently associated with three, underlying gp41 components in a meta-stable configuration whose fusion potential is triggered by interaction with cell surface receptors. This pre-fusion form may optimally present neutralization epitopes. We refer to this form of the envelope glycoproteins as native gp120-gp41. However, other oligomeric forms are possible, and it is important to define these (see Fig. 1).

Gp160: The full-length gp160 molecule often aggregates when expressed as a recombinant protein, at least in part because it contains the hydrophobic transmembrane domain. One such molecule is derived from a natural mutation that prevents the processing of the gp160 precursor to gp120/gp41 (VanCott et al J Virol 71: 4319, 1997). The gp160 precursor does not mediate virus-cell fusion and is a poor mimic of fusion-competent gp120/gp41. When evaluated in humans, recombinant gp160 molecules offered no advantages over gp120 monomers (Gorse et al., Vaccine 16: 493, 1998).

Uncleaved gp140 (gp140UNC): Stable Aoligomers@ have been made by eliminating the natural proteolytic site needed for conversion of the gp160 precursor protein into gp120 and gp41 (Berman et al, J Virol. 63: 3489, 1989; Earl et al Proc. Natl Acad Sci 87: 648, 1990). To express these constructs as soluble proteins, a stop codon is inserted within the *env* gene to truncate the protein immediately prior to the transmembrane-spanning segment of gp41. The protein lacks the transmembrane domain and the long, intracytoplasmic tail of gp41, but retains the regions important for virus entry and the induction of neutralizing Abs. The secreted protein contains full-length gp120 covalently linked through a peptide bond to the ectodomain of gp41. The protein migrates in SDS-PAGE as a single species with an apparent molecular mass of approximately 140 kilodaltons (kDa) under both

reducing and nonreducing conditions. The protein forms higher molecular weight noncovalent oligomers, likely through interactions mediated by the gp41 moieties.

Several lines of evidence suggest that the uncleaved gp140 molecule does not adopt the same conformation as native gp120-gp41. These include observations described herein and from the finding that uncleaved gp120-gp41 complexes do not avidly bind fusion co-receptors (R. Doms, personal communication). Furthermore, a gp140 protein of this type was unable to efficiently select for neutralizing MAbs when used to pan a phage-display library, whereas virions were efficient (Parren et al, J Virol. 70:9046, 1996). We refer to the uncleaved gp120-gp41 ectodomain material as gp140UNC.

Cleavable but uncleaved gp140 (gp140NON):
During biosynthesis, gp160 is cleaved into gp120 and gp41 by a cellular endoprotease of the furin family. Mammalian cells have a finite capacity to cleave gp120 from gp41, as we show below. Thus, when over-expressed, the envelope glycoproteins can saturate the endogenous furin enzymes and be secreted in precursor form. Since these molecules are potentially cleavable, we refer to them as gp140NON. Like gp140UNC, gp140NON migrates in SDS-PAGE with an apparent molecular mass of approximately 140 kDa under both reducing and nonreducing conditions. As shown below, gp140NON appears to possess the same non-native topology as gp140UNC.

Cleaved gp140 (gp140CUT): gp140CUT refers to full-length gp120 and ectodomain gp41 fully processed and capable of forming oligomers as found on virions. The noncovalent interactions between gp120 and gp41 are sufficiently long-lived for the virus to bind and initiate fusion with new target cells, a process which is likely completed within minutes during natural infection. The association has, however, to date proven too

labile for the production of significant quantities of cleaved gp140s in near homogenous form.

Stabilization of viral envelope glycoproteins

The metastable pre-fusion conformation of viral envelope proteins such as gp120/gp41 has evolved to be sufficiently stable so as to permit the continued spread of infection yet sufficiently labile to readily allow the conformational changes required for virus-cell fusion. For the HIV isolates examined thus far, the gp120-gp41 interaction has proven too unstable for preparative-scale production of gp140CUT as a secreted protein. Given the enormous genetic diversity of HIV, however, it is conceivable that viruses with superior env stability could be identified using screening methods such as those described herein. Alternatively, viruses with heightened stability could in principle be selected following successive exposure of virus to conditions known to destabilize the gp120-gp41 interaction. Such conditions might include elevated temperatures in the range of 37-60 EC and/or low concentrations of detergents or chaotropic agents. The envelope proteins from such viruses could be subcloned into the pPPI4 expression vector and analyzed for stability using our methods as well.

One could also adopt a semi-empirical, engineered approach to stabilizing viral envelope proteins. For example stable heterodimers have been successfully created by introducing complementary "knob" and "hole" mutations in the binding partners (Atwell *et al.*, J. Mol. Biol. 4:26, 1997). Alternatively or in addition, one could introduce other favorable interactions, such as salt bridges, hydrogen bonds, or hydrophobic interactions. This approach is facilitated by increased understanding of the structures of the SU and TM proteins, and the results described herein contribute to this understanding.

As we demonstrate in this invention, SU-TM stabilization can also

be achieved by means of one or more introduced disulfide bonds. Among mammalian retroviruses, only the lentiviruses such as HIV have non-covalent associations between the surface (SU) and transmembrane (TM) glycoproteins. In contrast, the type C and type D retroviruses all have an inter-subunit disulfide bond. The ectodomains of retroviral TM glycoproteins have a broadly common structure, one universal feature being the presence of a small, Cys-Cys bonded loop approximately central in the ectodomain. In the type C and D retroviral TM glycoproteins, an unpaired cysteine residue is found immediately C-terminal to this loop and is almost certainly used in forming the SU-TM disulfide bond. (Gallaher et al, AIDS Res Human Retrovir 11: 191, 1995; Schultz et al AIDS Res Human Retrovir, 8: 1585, 1992).

Although gp41 and other lentiviral TM glycoproteins lack the third cysteine, the structural homologies suggest that one could be inserted in the vicinity of the short central loop structure. Thus there is strong mutagenic evidence that the first and last conserved regions of gp120 (C1 and C5 domains) are probable contact sites for gp41.

The subject invention provides isolated nucleic acid molecules that encode mutant viral surface and transmembrane proteins in stabilized, antigenically authentic forms. This invention describes the design and synthesis of the stabilized viral proteins. Importantly, when appropriate methods are used to effect the stabilization, the viral proteins adopt conformations with desirable features. The subject invention further provides protein- or nucleic acid-based vaccines comprising mutant viral envelope proteins, antibodies isolated or identified using mutant viral envelope proteins, pharmaceutical compositions comprising these vaccines or antibodies, and methods of using these compositions to treat or prevent infections from viruses such as

HIV. The invention describes applications of the mutant viral proteins to identify whether a compound is capable of inhibiting a virus, and compounds identified in this manner.

Summary of the Invention

This invention provides an isolated nucleic acid which comprises a nucleotide segment having a sequence encoding a viral envelope protein comprising a viral surface protein and a corresponding viral transmembrane protein wherein the viral envelope protein contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and the viral transmembrane protein.

This invention provides an isolated nucleic acid which comprises a nucleotide segment having a sequence encoding a mutant viral envelope protein which differs from the corresponding wild type viral envelope protein sequence in at least one amino acid which upon proteolysis yields a complex comprising a surface protein and a transmembrane protein which has enhanced stability relative to the corresponding complex obtained from the wild type envelope protein.

In one embodiment of the above the viral surface protein is HIV-1 gp120 or a modified form of gp120 which has modified immunogenicity relative to wild type gp120. In one embodiment, the transmembrane protein is HIV-1 gp41 or a modified form of gp41 which has modified immunogenicity relative to wild type gp41.

This invention provides a vaccine which comprises the above isolated nucleic acid. In one embodiment, the vaccine comprises a therapeutically effective amount of the nucleic acid. In another embodiment, the vaccine comprises a therapeutically effective amount of the protein encoded by the above nucleic acid. In another embodiment, the vaccine comprises a combination of the recombinant nucleic acid molecule and the mutant viral envelope protein.

This invention provides a method of treating a viral disease which comprises immunizing a virally infected subject with the above vaccines or a combination thereof, thereby treating the subject.

This invention provides a vaccine which comprises a prophylactically effective amount of the above isolated nucleic acid.

This invention provides a vaccine which comprises a prophylactically effective amount of the protein encoded by the above isolated nucleic acid.

This invention provides a method of reducing the likelihood of a subject becoming infected with a virus comprising administering the above vaccines or a combination thereof, thereby reducing the likelihood of the subject becoming infected with the virus.

This invention provides the above vaccine which comprises but is not limited to the following: a recombinant subunit protein, a DNA plasmid, an RNA molecule, a replicating viral vector, a non-replicating viral vector, or a combination thereof.
This invention provides a method of reducing the severity of a viral disease in a subject comprising administering the above vaccine or a combination thereof, prior to exposure of the subject to the virus, thereby reducing the severity of the viral disease in the subject upon subsequent exposure to the virus.

This invention provides a mutant viral envelope protein which differs from the corresponding wild type protein in at least one amino acid which upon proteolysis yields a complex comprising a surface protein and a transmembrane protein which has enhanced stability relative to the corresponding complex obtained from the wild type envelope protein.

This invention provides a complex comprising a viral surface protein and a viral transmembrane protein which has enhanced stability relative to the corresponding complex obtained from the wildtype envelope protein, yielded by the proteolysis of a mutant viral envelope protein with a sequence which differs from the corresponding wild type protein sequence in at least one amino acid.

This invention provides a mutant viral envelope protein which is encoded by the above nucleic acid molecule.

This invention provides a vaccine which comprises a therapeutically effective amount of the above protein or complex. This invention also provides a vaccine which comprises a prophylactically effective amount of the above protein or complex.

This invention provides a method of stimulating or enhancing in a subject production of antibodies which recognize the above protein or complex.

This invention provides a method of stimulating or enhancing in a subject the production of cytotoxic T lymphocytes which recognize the above protein.

This invention provides an antibody capable of specifically binding to the above mutant protein. This invention also provides an antibody which is capable of specifically binding to the above mutant protein or complex but not to the wild type protein or complex.

This invention provides an antibody, antibody chain or fragment thereof identified using the viral envelope protein encoded by the above recombinant nucleic acid molecule. The antibody may be of the IgM, IgA, IgE or IgG class or subclasses thereof. The

above antibody fragment includes but is not limited to Fab, Fab=, (Fab=)2, Fv and single chain antibodies.

This invention provides an isolated antibody light chain of the above antibody, or fragment or oligomer thereof. This invention also provides an isolated antibody heavy chain of the above antibody, or fragment or oligomer thereof. This invention also provides one or more CDR regions of the above antibody. In one embodiment, the antibody is derivatized. In another embodiment, the antibody is a human antibody. The antibody includes but is not limited to monoclonal antibodies and polyclonal antibodies. In one embodiment, antibody is humanized.

This invention provides an isolated nucleic acid molecule encoding the above antibody.

This invention provides a method of reducing the likelihood of a virally exposed subject from becoming infected with the virus comprising administering the above antibody or the above isolated nucleic acid, thereby reducing the likelihood of the subject from becoming infected with the virus.

This invention provides a method of treating a subject infected with a virus comprising administering the above antibody or the above isolated nucleic acid, thereby treating the subject. In a preferred embodiment, the virus is HIV.

This invention provides an agent capable of binding the mutant viral envelope protein encoded by the above recombinant nucleic acid molecule. In one embodiment, the agent inhibits viral infection.

This invention provides a method for determining whether a compound is capable of inhibiting a viral infection comprising:

(A) contacting an appropriate concentration of the compound

with the mutant viral envelope protein encoded by the recombinant nucleic acid of claim 1 under conditions permitting binding of the compound to said protein;

(B) contacting the resulting complex with a reporter molecule under conditions that permit binding of the reporter molecule to the mutant viral envelope protein;

(C) measuring the amount of bound reporter molecule; and

(D) comparing the amount of bound reporter molecule in step (C) with the amount determined in the absence of the compound, a decrease in the amount indicating that the compound is capable of inhibiting infection by the virus, thereby determining whether a compound is capable of inhibiting a viral infection.

This invention provides a method for determining whether a compound is capable of inhibiting a viral infection which comprises:

(a) contacting an appropriate concentration of the compound with a host cell viral receptor or molecular mimic thereof under conditions that permit binding of the compound and receptor or receptor mimic;

(b) contacting the resulting complex with the mutant viral envelope protein encoded by the recombinant nucleic acid of claim 1 under conditions that permit binding of the envelope protein and receptor or receptor mimic in the absence of the compound;

(c) measuring the amount of binding of envelope protein to receptor or receptor mimic;

(d) comparing the amount of binding determined in step (c) with the amount determined in the absence of the compound, a decrease in the amount indicating that the compound is capable of inhibiting infection by the virus, thereby determining whether a compound is capable of inhibiting a viral infection.

This invention further provides a simple method for determining whether a subject has produced antibodies capable of blocking the infectivity of a virus.

This invention provides the above method wherein the compound was not previously known.

This invention provides a compound determined to be capable of inhibiting a viral infection by the above methods.

This invention provides a pharmaceutical composition comprising an amount of the compound effective to inhibit viral infection determined by the above methods to be capable of inhibiting viral infection and a pharmaceutically acceptable carrier. In one embodiment, wherein the viral infection is HIV-1 infection. In the preferred embodiment, the virus is HIV.

This invention provides a mutant viral envelope protein which differs from the corresponding wild type protein in at least one amino acid which yields a complex comprising a surface protein and a transmembrane protein which has enhanced stability relative to the corresponding complex obtained from the wild type envelope protein, wherein the surface protein and transmembrane protein are encoded by different nucleic acids.

This invention provides a complex comprising a viral surface protein and a viral transmembrane protein which has enhanced stability relative to the corresponding complex obtained from the wildtype envelope protein, yielded by the proteolysis of a mutant viral envelope protein with a sequence which differs from the corresponding wild type protein sequence in at least one amino acid, wherein the surface protein and transmembrane protein are encoded by different nucleic acids.

This invention provides an antibody which binds to the above

protein or above complex but does not cross react with the individual monomeric surface protein or the individual monomeric transmembrane protein. This invention provides the above antibody capable of binding to the HIV-1 virus.

Brief Description of the Figures

Figure 1.

Different forms of the HIV-1 envelope glycoproteins

The cartoons depict: i) Monomeric gp120; ii) Full-length recombinant gp160 iii) Proteolytically unprocessed gp140 trimer with the peptide bond maintained between gp120 and gp41 (gp140UNC or gp140NON); iv) The SOS gp140 protein, a proteolytically processed gp140 stabilized by an intermolecular disulfide bond; v) Native, virion-associated gp120-gp41 trimer. The shading of the gp140UNC protein (iii) indicates the major antibody-accessible regions that are poorly, or not, exposed on the SOS gp140 protein or on the native gp120-gp41 trimer.

Figure 2.

Co-transfection of furin increases the efficiency of cleavage of the peptide bond between gp120 and gp41

293T cells were transfected with DNA expressing HIV-1 JR-FL gp140WT or gp140UNC(gp120-gp41 cleavage-site mutant) proteins, in the presence or absence of a co-transfected furin-expressing plasmid. The 35S-labelled envelope glycoproteins secreted from the cells were immunoprecipitated with the anti-gp120 MAb 2G12, then analyzed by SDS-PAGE. Lane 1, gp140WT(gp140/gp120 doublet); Lane 2, gp140WT plus furin (gp120 only); Lane 3, gp140UNC (gp140 only); lane 4, gp140UNC plus furin (gp140 only). The approximate molecular weights, in kDa, of the major species are indicated on the left.

Figure 3.

Positions of cysteine substitutions in JR-FL gp140

The various residues of the JR-FL gp140WT protein that have been mutated to cysteines in one or more mutants are indicated by closed arrows on the schematics of the gp120 and gp41ECTO subunits. The positions of the alanine-492 and threonine-596

residues that are both mutated to cysteine in the SOS gp140 protein are indicated by the larger, closed arrows. a)JR-FL gp120. b) JR-FL gp41. The open boxes at the C-terminus of gp120 and the N-terminus of gp41 indicate the regions that are mutated in the gp140UNC protein to eliminate the cleavage site between gp120 and gp41.

Figure 4.

Immunoprecipitation analysis of selected double cysteine mutants of JR-FL gp140

The 35S-labelled envelope glycoproteins secreted from transfected 293T cells were immunoprecipitated with anti-gp120 and anti-gp41 MAbs, then analyzed by SDS-PAGE. The MAbs used were either 2G12 (anti-gp120 C3-V4 region) or F91 (anti-gp120 CD4 binding site region).

The positions of the two cysteine substitutions in each protein (one in gp120, the other in gp41ECTO) are noted above the lanes. The gp140WT protein is shown in lane 15. All proteins were expressed in the presence of co-transfected furin, except for the gp140WT protein.

Figure 5.

The efficiency of intermolecular disulfide bond formation is dependent upon the positions of the cysteine substitutions The 35S-labelled envelope glycoproteins secreted from 293T cells co-transfected with furin and the various gp140 mutants were immunoprecipitated with the anti-gp120 MAb 2G12, then analyzed by SDS-PAGE. For each mutant, the intensities of the 140kDa and 120kDa bands were determined by densitometry and the gp140/gp140+gp120 ratio was calculated and recorded. The extent of shading is proportional to the magnitude of the gp140/gp140+gp120 ratio. The positions of the amino acid

substitutions in gp41 and the C1 and C5 domains of gp120 are recorded along the top and down the sides, respectively. N.D. = Not done.

Figure 6.

Confirmation that an intermolecular gp120-gp41 bond forms in the SOS gp140 protein

293T cells were transfected with plasmids expressing gp140 proteins and, when indicated, a furin-expressing plasmid. The secreted, 35S-labelled glycoproteins were immunoprecipitated with the indicated MAbs and analyzed by SDS-PAGE under reducing (+DTT) or nonreducing conditions.

A. Radioimmunoprecipitations with 2G12 of the SOS gp140, gp140WT and gp140UNC proteins. Immunoprecipitated proteins were resolved by SDS-PAGE under reducing (Lanes 4-6) or non-reducing (Lanes 1-3) conditions.

B. Radioimmunoprecipitations with 2G12 of the SOS gp140 protein and gp140 proteins containing the corresponding single-cysteine mutations. 140kDa protein bands are not observed for either the A492C or the T596C single-cysteine mutant gp140 proteins.

C. Radioimmunoprecipitations with 2G12 of the SOS gp140 proteins produced in the presence or absence of co-transfected furin. Immunoprecipitated proteins were resolved by SDS-PAGE under reducing (Lanes 3-4) or non-reducing (Lanes 1-2) conditions. DTT is shown to reduce the 140 kDa SOS protein band produced in the presence but not the absence of exogenous furin.

Figure 7.

Analysis of cysteine mutants of JR-FL gp140

The 35S-labelled envelope glycoproteins secreted from transfected 293T cells were immunoprecipitated with the anti-gp120 MAb 2G12, then analyzed by SDS-PAGE. All gp140s were expressed in the presence of co-transfected furin. Lanes 1-8, gp140s containing the indicated double cysteine mutations. Lanes 9-11, gp140

proteins containing the A492C/T596C double cysteine substitutions together with the indicated lysine to alanine substitutions at residue 491 (lane 9), residue 493 (lane 10) or at both residues 491 and 493 (lane 11). Lanes 12-14, gp140 proteins containing quadruple cysteine substitutions.

Figure 8.

Comparison of the antigenic structures of the SOS gp140, W44C/T596C gp140 mutant, gp140UNC and gp140WT proteins

The 35S-labelled envelope glycoproteins secreted from transfected 293T cells were immunoprecipitated with the indicated anti-gp120 Mabs and anti-gp41 MAbs, then analyzed by SDS-PAGE. Mutant but not wild type gp140s were expressed in the presence of cotransfected furin.

A. Anti-gp120 immunoglobulins that neutralize HIV-1_{JR-FL}.

B. Non-neutralizing antibodies to the C1, C4 and C5 regions of gp120.

C. Antibodies to CD4-induced epitopes were examined alone and in combination with sCD4.

D. Neutralizing (2F5) and non-neutralizing (7B2, 2.2B and 25C2) anti-gp41 antibodies and MAb 2G12.

E. Radioimmunoprecipitations of gp140WT (odd numbered lanes) and gp140UNC (even numbered lanes).

Figure 9

Preparation of disulfide bond-stabilized gp140 proteins from various HIV-1 isolates

293T cells were transfected with plasmids expressing wild type or mutant gp140s in the presence or absence of exogenous furin as indicated. 35S-labeled supernatants were prepared and analyzed by radioimmunoprecipitation with MAb 2G12 as described above. Lane 1: SOS gp140 protein. Lane 2: gp140WT plus furin. Lane 3: gp140WT without furin. (A)HIV-1 DH123. (B)HIV-1 HxB2

Figure 10

Amino acid sequences of the glycoproteins with various deletions in the variable regions. The deleted wild-type sequences are shown in the white shade and include the following: ΔV1: D132-K152; Δ2: F156-I191; ΔV1V2': D132-K152 and F156-I191; ΔV1V2*: V126-S192; ΔV3: N296-Q324

Figure 11

Formation of an intersubunit cysteine bridge in envelope proteins with deletions in variable loop regions. a) The ΔV1V2*V3 protein and the ΔV1V2*V3 N357Q N398Q protein with two cysteines at positions 492 and 596 (indicated with CC) were precipitated with 2G12 and F91 (lanes 3 & 7 and 4 & 8, respectively). The appropriate controls without cysteine mutations are shown in lanes 1, 2, 5 & 6. The wild-type protein without extra cysteines is shown in lanes 9 and 10. All the proteins were cleaved by furin, except for the wild-type protein of lane 10. The approximate sizes in kDa are given on the right. b) Various loop deleted proteins with two cysteines at positions 492 and 596 (CC) were precipitated with 2G12 (lanes 3, 5, 7, 9, 11 & 13). Proteins with the same deletions without extra cysteines are given in the adjacent lanes. These control proteins were not cleaved by furin. The full-length SOS gp140 protein is included as a control in lane 1.

Figure 12

Antigenic characterization of the A492C/T596C mutant in combination with deletions in the variable loops. All mutants were expressed in the presence of exogenous furin. The Abs used in RIPAs are indicated on top. a) The A492C/T596C ΔV1V2* mutant and b) the A492C/T596C ΔV3 mutant.

Figure 13

Nucleotide (A) and amino acid (B) sequences for HIV-1_{JR-FL} SOS gp140. The amino acid numbering system corresponds to that for

wild-type JR-FL (Genbank Accession #U63632). The cysteine mutations are indicated in underlined bold type face.

Figure 14
Nucleotide (A) and amino acid (B) sequences for HIV-1_{JR-FL} ΔV1V2* SOS gp140. The amino acid numbering system corresponds to that for wild-type JR-FL (Genbank Accession #U63632). The cysteine mutations are indicated in underlined bold type face.

Figure 15
Nucleotide (A) and amino acid (B) sequences for HIV-1_{JR-FL} ΔV3 SOS gp140. The amino acid numbering system corresponds to that for wild-type JR-FL (Genbank Accession #U63632). The cysteine mutations are indicated in underlined bold type face.

Figure 16
SDS-PAGE analysis of purified HIV-1_{JR-FL} SOS gp140, $gp140_{UNC}$ and gp120 proteins. CHO cell-expressed proteins (0.5 μg) in Laemmli sample buffer with (reduced) or without (non-reduced) 50 mM DTT were resolved on a 3-8% polyacrylamide gradient gel.

Figure 17
Biophysical analyses of purified, CHO cell-expressed HIV-1_{JR-FL} envelope glycoproteins. (A) Ultracentrifugation analysis of SOS gp140 was performed at protein concentrations ranging from 0.25 mM to 1.0 mM. The experimental data (open circles) were compared with theoretical curves for ideal monomers, dimers and trimers (labeled 1, 2, 3). (B) Analytical size exclusion chromatography. Purified SOS gp140, $gp140_{UNC}$ and gp120 proteins were resolved on a TSK $G3000SW_{XL}$ column in PBS buffer, and their retention times were compared with those of known m. wt. standard proteins of 220 kDa, 440 kDa and 880 kDa (arrowed). The main peak retention time of SOS gp140 (5.95 min) is consistent with it being a monomer that is slightly larger than monomeric gp120 (retention time 6.24 min), whereas $gp140_{UNC}$ (retention time 4.91 min) migrates as

oligomeric species. (C) The oligomeric status of pure standard proteins, thyroglobulin, ferritin and albumin, were compared with gp120 and gp120 in complex with soluble CD4 using BN-PAGE. The proteins were visualized on the gel using coomassie blue. (D) BN-PAGE analysis of CHO cell-derived, purified HIV-1_{JR-FL} gp120, SOS gp140 and $gp140_{UNC}$ glycoproteins.

Figure 18

Negative stain electron micrographs of SOS gp140 alone (A) and in complex with MAbs (B-F). Bar = 40 nm. In B-F, the panels were masked and rotated so that the presumptive Fc of the MAb is oriented downward. When multiple MAbs were used, the presumptive Fc of MAb 2F5 is oriented downward. In B-F, interpretative diagrams are also provided to illustrate the basic geometry and stoichiometry of the immune complexes. SOS gp140, intact MAb, and $F(ab')_2$ are illustrated by ovals, Y-shaped structures and V-shaped structures, respectively, in the schematic diagrams, which are not drawn to scale. The MAbs used are as follows: (B) 2F5; (C) IgG1b12; (D) 2G12; (E) MAb 2F5 plus $F(ab')_2$ IgG1b12; (F) MAb 2F5 plus MAb 2G12.

Figure 19

Individual, averaged and subtracted electron micrographs of SOS gp140 and gp120 in complex with sCD4 and MAb 17b. Bar = 40 nm. Panels A and B are individual electron micrographs of ternary complexes of SOS gp140 (A) and YU2 gp120 (B). The Fc region of MAb 17b is aligned downward. Panels C and F are averaged electron micrographs of ternary complexes of SOS gp140 (C) and gp120 (F). Panels D and G are masked and averaged electron micrographs of the SOS gp140 complex (D) and the gp120 complex (G). Panel E represents the density remaining upon subtraction of the gp120 complex (Panel G) from the gp140 complex (Panel D). In (D) and (E), the arrow indicates the area of greatest residual density, which represents the presumptive $gp41_{ECTO}$ moiety that is present in SOS gp140 but not in gp120. Panel H indicates the outline of

the gp120 complex (Panel G) overlaid upon a ribbon diagram of the X-ray crystal structure of the gp120 core in complex with sCD4 and the 17b Fab fragment [PDB code 1GC1](Kwong, P.D., et al., Nature 393:648-659 (1998)). The gp120 complex was enlarged to facilitate viewing.

Figure 20

Models indicating the approximate location of $gp41_{ECTO}$ in relation to gp120 as derived from electron microscopy data of SOS gp140. (A) Presumptive location of $gp41_{ECTO}$ (represented by the dark blue oval) in relation to the X-ray crystal structure of the gp120 core in complex with sCD4 (yellow) and Fab 17b (light blue) [PDB code 1GC1](Kwong, P.D., et al., Nature 393:648-659 (1998)). The gp120 core surface was divided into three faces according to their antigenic properties (Moore, J.P. et al., J.Virol. 70 :1863-1872 (1996); ,Wyatt, R., et al., Nature 393:705-711 (1998)): the non-neutralizing face is colored lavender, the neutralizing face is red, and the silent face, green. The IgG1b12 epitope (Saphire, E.O., et al., Science 293:1155-1159 (2001)) and the 2G12 epitope (Wyatt, R., et al., Nature 393:705-711 (1998)) are shown in yellow and white, respectively. The residues associated with the gp120 C-terminus are colored blue, to provide a point of reference.

Figure 21

RIPA analysis of unpurified, CHO cell-expressed $HIV\text{-}1_{JR\text{-}FL}$ SOS gp140. Stably transfected CHO cells were cultured in the presence of ^{35}S-labeled cysteine and methionine. Culture supernatants were immunoprecipitated with the indicated MAbs and protein G-agarose beads, and bound proteins were resolved by SDS-PAGE and visualized by autoradiography. The MAb and/or CD4-based protein used for capture is indicated above each lane. In Lane 2, the proteins were reduced with DTT prior to SDS-PAGE; the remaining samples were analyzed under non-reducing conditions.

Figure 22

SPR analysis of CHO cell-expressed HIV-1_{JR-FL} SOS gp140, $gp140_{UNC}$ and gp120 proteins. Anti-gp120 and anti-gp41 MAbs were immobilized onto sensor chips and exposed to buffers containing the indicated gp120 or gp140 glycoproteins in either purified or unpurified form, as indicated. Where noted, Env proteins were mixed with an 8-fold molar excess of sCD4 for 1h prior to analysis. Culture supernatants from stably transfected CHO cells were used as the source of unpurified SOS gp140 and $gp140_{UNC}$ proteins. The concentrations of these proteins were measured by Western blotting and adjusted so that approximately equal amounts of each protein were loaded. Only the binding phases of the sensorgrams are shown; in general, the dissociation rates were too slow to provide meaningful information.

Figure 23

BN-PAGE analyses of unfractionated cell culture supernatants. (A) Comparison of HIV-1_{JR-FL} gp120, SOS gp140, $gp140_{UNC}$, and ΔV1V2 SOS gp140 glycoproteins present in culture supernatants from stable CHO cell lines. (B) Proteolytic cleavage destabilizes gp140 oligomers. 293T cells were transfected with furin and plasmids encoding SOS gp140, $gp140_{UNC}$, SOS $gp140_{UNC}$. Cell culture supernatants were combined with MOPS buffer containing 0.1% coomassie blue and resolved by BN-PAGE. Proteins were then transferred to PVDF membranes and visualized by Western blotting. Thyroglobulin and the BSA dimer were used as molecular weight markers (see Fig. 2C).

Detailed Description of the Invention

This invention provides an isolated nucleic acid which comprises a nucleotide segment having a sequence encoding a viral envelope protein comprising a viral surface protein and a corresponding viral transmembrane protein wherein the viral envelope protein contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and transmembrane protein.

This invention provides an isolated nucleic acid which comprises a nucleotide segment having a sequence encoding a mutant viral envelope protein which differs from the corresponding wild type viral envelope protein sequence in at least one amino acid which upon proteolysis yields a complex comprising a surface protein and a transmembrane protein which has enhanced stability relative to the corresponding complex obtained from the wild type envelope protein.

As used herein, "enhance the stability" means make more long-lived or resistant to dissociation. The interaction may be stabilized by the introduction of disulfide bonds, salt bridges, hydrogen bonds, hydrophobic interactions, favorable van der Waals contacts, a linker peptide or a combination thereof. The stabilizing interactions may be introduced by recombinant methods. Alternatively or in combination, stabilized viral envelope proteins may be obtained by selection methods such as exposing a virus to conditions known to destabilize the interaction between the surface and transmembrane envelope proteins, and then selecting for resistant viruses. This process may be repeated one or more times until one obtains viral envelope proteins with the desired stability. Alternatively, one may screen isolates for naturally occurring mutations that enhance the stability of the interaction between the surface and transmembrane proteins, relative to the stability observed for

prototypic wild type viral envelope proteins.

The invention does not encompass known viral proteins wherein the endoproteolytic processing of the precursor envelope protein to separate surface and transmembrane proteins is prevented by expressing the protein in the absence of sufficient quantities of the endoprotease or by mutating the endoproteolytic cleavage site in the absence of additional mutations, such as the addition of a linker peptide. In such known viral envelope proteins, the viral surface and transmembrane proteins are physically joined by a covalent bond but are not known to form a complex, as illustrated in Figure 1.

One embodiment of the above virus is a lentivirus. In one embodiment, the virus is the simian immunodeficiency virus. Another embodiment of the above virus is the human immunodeficiency virus (HIV). The virus may be either of the two known types of HIV (HIV-1 or HIV-2). The HIV-1 virus may represent any of the known major subtypes (Clades A, B, C, D E, F, G and H) or outlying subtype (Group O). Additional types, subtypes or classes of HIV may be discovered and used in this invention. In one embodiment, the human immunodeficiency virus is a primary isolate. In one embodiment, the human immunodeficiency virus is HIV-1_{JR-FL}. In another embodiment the human immunodeficiency virus is HIV-1_{DH123}. In another embodiment the human immunodeficiency virus is HIV-1_{Gun-1}. In another embodiment the human immunodeficiency virus is HIV-$1_{89.6}$. In another embodiment the human immunodeficiency virus is HIV-1_{HXB2}.

HIV-1_{JR-FL} is a strain that was originally isolated from the brain tissue of an AIDS patient taken at autopsy and co-cultured with lectin-activated normal human PBMCs (O=Brien et al, Nature, 348: 69, 1990) HIV-1_{JR-FL} is known to utilize CCR5 as a fusion coreceptor and has the ability to replicate in phytohemagglutinin (PHA)-stimulated PBMCs and blood-derived macrophages but does not

replicate efficiently in most immortalized T cell lines.

$HIV\text{-}1_{DH123}$ is a clone of a virus originally isolated from the peripheral mononuclear cells (PBMCs) of a pateint with AIDS (Shibata et al., J. Virol 69:4453, 1995). $HIV\text{-}1_{DH123}$ is known to utilize both CCR5 and CXCR4 as fusion coreceptors and has the ability to replicate in PHA-stimulated PBMCs, blood-derived macrophages and immortalized T cell lines.

$HIV\text{-}1_{Gun\text{-}1}$ is a cloned virus originally isolated from the peripheral blood mononuclear cells of a hemophilia B patient with AIDS (Takeuchi et al., Jpn J Cancer Res 78:11 1987). $HIV\text{-}1_{Gun\text{-}1}$ is known to utilize both CCR5 and CXCR4 as fusion coreceptors and has the ability to replicate in PHA-stimulated PBMCs, blood-derived macrophages and immortalized T cell lines.

$HIV\text{-}1_{89.6}$ is a cloned virus originally isolated from a patient with AIDS (Collman et al, J. Virol. 66: 7517, 1992). $HIV\text{-}1_{89.6}$ is known to utilize both CCR5 and CXCR4 as fusion coreceptors and has the ability to replicate in PHA-stimulated PBMCs, blood-derived macrophages and immortalized T cell lines.

$HIV\text{-}1_{HXB2}$ is a TCLA virus that is known to utilize CXCR4 as a fusion coreceptor and has the ability to replicate in PHA-stimulated PBMCs and immortalized T cell lines but not blood derived macrophages.

Although the above strains are used herein to generate the mutant viral envelope proteins of the subject invention, other HIV-1 strains could be substituted in their place as is well known to those skilled in the art.

One embodiment of the above viral surface protein is gp120 or a modified form of gp120 which has modified immunogenicity relative to wild type gp120. In one embodiment, the modified gp120

molecule is characterized by the absence of one or more variable loops present in wild type gp120. In one embodiment, the variable loop comprises V1, V2, or V3. In one embodiment, the modified gp120 molecule is characterized by the absence or presence of one or more canonical glycosylation sites not present in wild type gp120. In one embodiment, one or more canonical glycosylation sites are absent from the V1V2 region of the gp120 molecule.

In one embodiment, the transmembrane protein is gp41 or a modified form of gp41 which has modified immunogenicity relative to wildtype gp41. In one embodiment, the transmembrane protein is full-length gp41. In another embodiment, the transmembrane protein contains the ectodomain and membrane anchoring sequence of gp41 but lacks a portion or all of the gp41 cytoplasmic sequences. In one embodiment, the transmembrane protein is the gp41 ectodomain. In one embodiment, the transmembrane protein is modified by deletion or insertion of one or more canonical glycosylation sites.

One embodiment of the above viral surface protein is gp120 or a derivative thereof. In one embodiment, the gp120 molecule has been modified by the deletion or truncation of one or more variable loop sequences. The variable loop sequences include but are not limited to V1, V2, V3 or a combination thereof. In another embodiment, the gp120 molecule has been modified by the deletion or insertion of one or more canonical glycosylation sites. The region of gp120 from which the canonical glycosylation sites are deleted includes but is not limited to the V1V2 region of the gp120 molecule.

The V1, V2 and V3 variable loop sequences for HIV-1_{JR-FL} are illustrated in Figure 10. The amino acid sequences in these variable loops will vary for other HIV isolates but will be located in homologous regions of the gp120 envelope glycoprotein.

As used herein, "canonical glycosylation site" includes but is not limited to an Asn-X-Ser or Asn-X-Thr sequence of amino acids that defines a site for N-linkage of a carbohydrate. In addition, Ser or Thr residues not present in such sequences to which a carbohydrate can be linked through an O-linkage are "canonical glycosylation sites." In the later case of a "canonical glycosylation site", a mutation of the Ser and Thr residue to an amino acid other than a serine or threonine will remove the site of O-linked glycosylation.

When used in the context of gp41, "derivatives" include but are not limited to the gp41 ectodomain, gp41 modified by deletion or insertion of one or more glycosylation sites, gp41 modified so as to eliminate or mask the well-known imunodominant epitope, a gp41 fusion protein, and gp41 labeled with an affinity ligand or other detectable marker.

As used herein, "ectodomain" means the extracellular region or portion thereof exclusive of the transmembrane spanning and cytoplasmic regions.

In one embodiment, the stabilization of the mutant viral envelope protein is achieved by the introduction of one or more cysteine-cysteine bonds between the surface and transmembrane proteins.

In one embodiment, one or more amino acids which are adjacent to or which contain an atom within 5 Angstroms of an introduced cysteine are mutated to a noncysteine residue.

As used herein, "Adjacent" to means immediately preceding or following in the primary sequence of the protein.

As used herein, "mutated" means that which is different from the wild-type.

As used herein, "noncysteine residue" means an amino acid other than cysteine.

In one embodiment, one or more cysteines in gp120 or modified form of gp120 are disulfide linked to one or more cysteines in gp41 or modified form of gp41.

In one embodiment, a cysteine in the C5 region of gp120 or modified form of gp120 is disulfide linked to a cysteine in the ectodomain of gp41 or modified form. In one embodiment, the disulfide bond is formed between a cysteine introduced by an A492C mutation in gp120 or modified form of gp120 and an T596C mutation in gp41 or modified form of gp41.

As used herein, "C5 region" means the fifth conserved sequence of amino acids in the gp120 glycoprotein. The C5 region includes the carboxy-terminal amino acids. In HIV-1_{JR-FL} gp120, the unmodified C5 region consists of the amino acids GGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQRE. Amino acid residues 462-500 of the sequence set forth in figure 3A have this sequence. In other HIV isolates, the C5 region will comprise a homologous carboxy-terminal sequence of amino acids of similar length.

As used herein, "A492C mutation" refers to a point mutation of amino acid 492 in HIV-1_{JR-FL} gp120 from alanine to cysteine. Because of the sequence variability of HIV, this amino acid will not be at position 492 in all other HIV isolates. For example, in HIV-1_{NL4-3} the corresponding amino acid is A499 (Genbank Accesion # AAA44992). It may also be a homologous amino acid other than alanine or cysteine. This invention encompasses cysteine mutations in such amino acids, which can be readily identified in other HIV isolates by those skilled in the art.

As used herein, "T596C mutation" refers to a point mutation of amino acid 596 in HIV-1_{JR-FL} gp41 from threonine to cysteine.

Because of the sequence variability of HIV, this amino acid will not be at position 596 in all other HIV isolates. For example, in HIV-1_{NL4-3} the corresponding amino acid is T603 (Genbank Accesion # AAA44992). It may also be a homologous amino acid other than threonine or cysteine. This invention encompasses cysteine mutations in such amino acids, which can be readily identified in other HIV isolates by those skilled in the art.

In another embodiment, a cysteine in the C1 region of gp120 is disulfide linked to a cysteine in the ectodomain of gp41.

As used herein, "C1 region" means the first conserved sequence of amino acids in the mature gp120 glycoprotein. The C1 region includes the amino-terminal amino acids. In HIV_{JR-FL}, the C1 region consists of the amino acids VEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPN PQEVVLENVTEHFNMWKNNMVEQMQEDIISLWDQSLKPCVKLTPLCVTLN. Amino acid resides 30-130 of the sequence set forth in in figure 3A have this sequence. In other HIV isolates, the C1 region will comprise a homologous amino-terminal sequence of amino acids of similar length. W44C and P600C mutations are as defined above for A492 and T596 mutations. Because of the sequence variability of HIV, W44 and P600 will not be at positions 44 and 600 in all HIV isolates. In other HIV isolates, homologous, non-cysteine amino acids may also be present in the place of the tryptophan and proline. This invention encompasses cysteine mutations in such amino acids, which can be readily identified in other HIV isolates by those skilled in the art.

The above isolated nucleic acid includes but is not limited to cDNA, genomic DNA, and RNA.

One skilled in the art would know how to make the nucleic acid which encode mutant viral envelope proteins wherein the

interaction between the viral surface and transmembrane proteins has been stabilized. Furthermore, one skilled in the art would know how to use these recombinant nucleic acid molecules to obtain the proteins encoded thereby, and practice the therapeutic and prophylactic methods of using same, as described herein for the recombinant nucleic acid molecule which encode mutant viral envelope proteins.

The invention provides a replicable vector comprising the above nucleic acid. This invention also provides a plasmid, cosmid, λ phage or YAC containing the above nucleic acid molecule. In one embodiment, the plasmid is designated PPI4. The invention is not limited to the PPI4 plasmid and may include other plasmids known to those skilled in the art.

In accordance with the invention, numerous vector systems for expression of the mutant glycoprotein may be employed. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow for the selection of transfected host cells. The marker may provide, for example, prototropy to an auxotrophic host, biocide resistance, (e.g., antibiotics) or resistance to heavy metals such as copper or the like. The selectable marker gene can be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcriptional promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by (Okayama and Berg, Mol Cell Biol 3:280, 1983).

The vectors used in the subject invention are designed to express high levels of mutant viral envelope proteins in cultured eukaryotic cells as well as efficiently secrete these proteins into the culture medium. The targeting of the mutant envelope glycoproteins into the culture medium is accomplished by fusing in-frame to the mature N-terminus of the mutant envelope glycoprotein a suitable signal sequence such as that derived from the genomic open reading frame of the tissue plasminogen activator (tPA).

The mutant envelope protein may be produced by a) transfecting a mammalian cell with an expression vector for producing mutant envelope glycoprotein; b) culturing the resulting transfected mammalian cell under conditions such that mutant envelope protein is produced; and c) recovering the mutant envelope protein so produced.

Once the expression vector or DNA sequence containing the constructs has been prepared for expression, the expression vectors may be transfected or introduced into an appropriate mammalian cell host. Various techniques may be employed to achieve this, such as, for example, protoplast fusion, calcium phosphate precipitation, electroporation, retroviral transduction, or other conventional techniques. In the case of protoplast fusion, the cells are grown in media and screened for the appropriate activity. Expression of the gene encoding a mutant envelope protein results in production of the mutant protein.

Methods and conditions for culturing the resulting transfected cells and for recovering the mutant envelope protein so produced are well known to those skilled in the art, and may be varied or optimized depending upon the specific expression vector and mammalian host cell employed.

In accordance with the claimed invention, the preferred host cells for expressing the mutant envelope protein of this invention are mammalian cell lines. Mammalian cell lines include, for example, monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line 293; baby hamster kidney cells (BHK); Chinese hamster ovary-cells-DHFR$^+$ (CHO); Chinese hamster ovary-cells DHFR$^-$ (DXB11); monkey kidney cells (CV1); African green monkey kidney cells (VERO-76); human cervical carcinoma cells (HELA); canine kidney cells (MDCK); human lung cells (W138); human liver cells (Hep G2); mouse mammary tumor (MMT 060562); mouse cell line (C127); and myeloma cell lines.

Other eukaryotic expression systems utilizing non-mammalian vector/cell line combinations can be used to produce the mutant envelope proteins. These include, but are not limited to, baculovirus vector/insect cell expression systems and yeast shuttle vector/yeast cell expression systems.

Methods and conditions for purifying mutant envelope proteins from the culture media are provided in the invention, but it should be recognized that these procedures can be varied or optimized as is well known to those skilled in the art.

This invention provides a host cell containing the above vector. In one embodiment, the cell is a eukaryotic cell. In another embodiment, the cell is a bacterial cell.

This invention provides a vaccine which comprises the above isolated nucleic acid. In one embodiment, the vaccine comprises a therapeutically effective amount of the nucleic acid. In another embodiment, the vaccine comprises a therapeutically effective amount of the protein encoded by the above nucleic acid. In another embodiment, the vaccine comprises a combination of the recombinant nucleic acid molecule and the mutant viral envelope protein.

Numerous adjuvants have been developed to enhance the immunogenicity of protein and/or nucleic acid vaccines. As used herein, adjuvants suitable for use with protein-based vaccines include, but are not limited to, alum, Freund's incomplete adjuvant (FIA), Saponin, Quil A, QS21, Ribi Detox, Monophosphoryl lipid A (MPL), and nonionic block copolymers such as L-121 (Pluronic; Syntex SAF). In a preferred embodiment, the adjuvant is alum, especially in the form of a thixotropic, viscous, and homogenous aluminum hydroxide gel. The vaccine of the subject invention may be administered as an oil in water emulsion. Methods of combining adjuvants with antigens are well known to those skilled in the art.

The adjuvant may be in particulate form. The antigen may be incorporated into biodegradable particles composed of poly-lactide-co-glycolide (PLG) or similar polymeric material. Such biodegradable particles are known to provide sustained release of the immunogen and thereby stimulate long-lasting immune responses to the immunogen. Other particulate adjuvants include but are not limited to a micellular mixture of Quil A and cholesterol known as immunostimulating complexes (ISCOMs) and aluminum or iron oxide beads. Methods for combining antigens and particulate adjuvants are well known to those skilled in the art. It is also known to those skilled in the art that cytotoxic T lymphocyte and other cellular immune responses are elicited when protein-based immunogens are formulated and administered with appropriate adjuvants, such as ISCOMs and micron-sized polymeric or metal oxide particles.

As used herein, suitable adjuvants for nucleic acid based vaccines include, but are not limited to, Quil A, interleukin-12 delivered in purified protein or nucleic acid form, short bacterial immunostimulatory nucleotide sequence such as CpG containing motifs, interleukin-2/Ig fusion proteins delivered in purified protein or nucleic acid form, oil in water micro-

emulsions such as MF59, polymeric microparticles, cationic liposomes, monophosphoryl lipid A (MPL), immunomodulators such as Ubenimex, and genetically detoxified toxins such as E. coli heat labile toxin and cholera toxin from Vibrio. Such adjuvants and methods of combining adjuvants with antigens are well known to those skilled in the art.

A "therapeutically effective amount" of the mutant envelope protein may be determined according to methods known to those skilled in the art.

As used herein, "therapeutically effective amount" refers to a dose and dosing schedule sufficient to slow, stop or reverse the progression of a viral disorder. In a preferred embodiment, the virus is HIV.

This invention provides a method of treating a viral disease which comprises immunizing a virally infected subject with the above vaccines or a combination thereof, thereby treating the subject.

As used herein, "treating" means either slowing, stopping or reversing the progression of a viral disorder. In the preferred embodiment, "treating" means reversing the progression to the point of eliminating the disorder. As used herein, "treating" also means the reduction of the number of viral infections, reduction of the number of infectious viral particles, reduction of the number of virally infected cells, or the amelioration of symptoms associated with the virus.

As used herein, "immunizing" means administering a primary dose of the vaccine to a subject, followed after a suitable period of time by one or more subsequent administrations of the vaccine, so as to generate in the subject an immune response against the vaccine. A suitable period of time between administrations of

the vaccine may readily be determined by one skilled in the art, and is usually on the order of several weeks to months.

Depending on the nature of the vaccine and size of the subject, the dose of the vaccine can range from about 1μg to about 10mg. In the preferred embodiment, the dose is about 300 μg.

As used herein, "virally infected" means the introduction of viral genetic information into a target cell, such as by fusion of the target cell membrane with the virus or infected cell. The target may be a bodily cell of a subject. In the preferred embodiment, the target cell is a bodily cell from a human subject.

As used herein, "subject" means any animal or artificially modified animal capable of becoming infected with the virus. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. The animals include but are not limited to mice, rats, dogs, guinea pigs, ferrets, rabbits, and primates. In the preferred embodiment, the subject is a human.

This invention provides a vaccine which comprises a prophylactically effective amount of the above isolated nucleic acid.

This invention provides a vaccine which comprises a prophylactically effective amount of the protein encoded by the above isolated nucleic acid.

A prophylactically effective amount of the vaccine may be determined according to methods well known to those skilled in the art.

As used herein "prophylactically effective amount" refers to a

dose and dosing schedule sufficient to reduce the likelihood of a subject becoming infected or to lessen the severity of the disease in subjects who do become infected.

This invention provides a method of reducing the likelihood of a subject becoming infected with a virus comprising administering the above vaccines or a combination thereof, thereby reducing the likelihood of the subject becoming infected with the virus.

As used herein, "the subject becoming infected with a virus" means the invasion of the subject's own cells by the virus.

As used herein, "reducing the likelihood of a subject's becoming infected with a virus" means reducing the likelihood of the subject's becoming infected with the virus by at least two-fold. For example, if a subject has a 1% chance of becoming infected with the virus, a two-fold reduction in the likelihood of the subject's becoming infected with the virus would result in the subject's having a 0.5% chance of becoming infected with the virus. In the preferred embodiment of this invention, reducing the likelihood of the subject's becoming infected with the virus means reducing the likelihood of the subject's becoming infected with the virus by at least ten-fold.

As used herein "administering" may be effected or performed using any of the methods known to one skilled in the art. The methods may comprise intravenous, intramuscular, oral, intranasal, transdermal or subcutaneous means.

This invention provides the above vaccine which comprises but is not limited to the following: a recombinant subunit protein, a DNA plasmid, an RNA molecule, a replicating viral vector, a non-replicating viral vector, or a combination thereof.

This invention provides a method of reducing the severity of a

viral disease in a subject comprising administering the above vaccine or a combination thereof, prior to exposure of the subject to the virus, thereby reducing the severity of the viral disease in the subject upon subsequent exposure to the virus. In the preferred embodiment, the virus is HIV.

As used herein "reducing the severity of a viral disease in a subject" means slowing the progression of and/or lessening the symptoms of the viral disease. It also means decreasing the potential of the subject to transmit the virus to an uninfected subject.

As used herein, "exposure to the virus" means contact with the virus such that infection could result.

As used herein, "subsequent exposure" means an exposure after one or more immunizations.

This invention provides a mutant viral envelope protein which differs from the corresponding wild type protein in at least one amino acid which upon proteolysis yields a complex comprising a surface protein and a transmembrane protein which has enhanced stability relative to the corresponding complex obtained from the wild type envelope protein.

This invention provides a complex comprising a viral surface protein and a viral transmembrane protein which has enhanced stability relative to the corresponding complex obtained from the wildtype envelope protein, yielded by the proteolysis of a mutant viral envelope protein with a sequence which differs from the corresponding wild type protein sequence in at least one amino acid.

This invention provides a viral envelope protein comprising a viral surface protein and a corresponding viral transmembrane

protein wherein the viral envelope protein contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and transmembrane protein.

This invention provides a complex comprising a viral surface protein and a corresponding viral transmembrane protein of a viral envelope protein wherein the viral envelope protein contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and transmembrane protein.

This invention provides a mutant viral envelope protein which is encoded by the above nucleic acid molecule.

In one embodiment, the mutant viral envelope protein is linked to at least one other protein or protein fragment to form a fusion protein.

This invention provides a virus-like particle which comprises the transmembrane protein and surface protein complex of the subject invention. In one embodiment, the virus-like particle comprises an immunodeficiency virus structural protein. In one embodiment, the structural protein is the gag protein.

As used herein, "virus-like particles" or VLPs are particle which are non-infectious in any host, nonreplicating in any host, which do not contain all of the protein components of live virus particles. As used herein, VLPs of the subject invention contain the disulfide-stabilized complex of the subject invention and a structural protein, such as HIV-1 gag, needed to form membrane-enveloped virus-like particles.

Advantages of VLPs include (1) their particulate and multivalent nature, which is immunostimulatory, and (2) their ability to

present the disulfide-stabilized envelope glycoproteins in a near-native, membrane-associated form.

VLPs are produced by co-expressing the viral proteins (e.g., HIV-1 gp120/gp41 and gag) in the same cell. This can be achieved by any of several means of heterologous gene expression that are well-known to those skilled in the art, such as transfection of appropriate expression vector(s) encoding the viral proteins, infection of cells with one or more recombinant viruses (e.g., vaccinia) that encode the VLP proteins, or retroviral transduction of the cells. A combination of such approaches can also be used. The VLPs can be produced either in vitro or in vivo.

VLPs can be produced in purified form by methods that are well-known to the skilled artisan, including centrifugation, as on sucrose or other layering substance,. and by chromatography.
As used herein, "mutant" means that which is not wild-type. As used herein, "linked" refers but is not limited to fusion proteins formed by recombinant methods and chemical cross links. Suitable chemical cross links are well known to those skilled in the art.

In one embodiment, the protein is purified by one of the methods known to one skilled in the art.

This invention provides a vaccine which comprises a therapeutically effective amount of the above protein or complex. This invention also provides a vaccine which comprises a prophylactically effective amount of the above protein or complex.

This invention provides a method of stimulating or enhancing in a subject production of antibodies which recognize the above protein or complex.

This invention provides a method of stimulating or enhancing in a subject the production of cytotoxic T lymphocytes which recognize the above protein.

This invention provides an antibody capable of specifically binding to the above mutant protein. This invention also provides an antibody which is capable of specifically binding to the above mutant protein or complex but not to the wild type protein or complex.

This invention provides an antibody, antibody chain or fragment thereof identified using the viral envelope protein encoded by the above recombinant nucleic acid molecule. The antibody may be of the IgM, IgA, IgE or IgG class or subclasses thereof. The above antibody fragment includes but is not limited to Fab, Fab', $(Fab')_2$, Fv and single chain antibodies. This invention provides a labeled antibody.

This invention provides an isolated antibody light chain of the above antibody, or fragment or oligomer thereof. This invention also provides an isolated antibody heavy chain of the above antibody, or fragment or oligomer thereof. This invention also provides one or more CDR regions of the above antibody. In one embodiment, the antibody is derivatized. In another embodiment, the antibody is a human antibody. The antibody includes but is not limited to monoclonal antibodies and polyclonal antibodies. In one embodiment, antibody is humanized.

As used herein "oligomer" means a complex of 2 or more subunits.

As used herein, "CDR" or complementarity determining region means a highly variable sequence of amino acids in the variable domain of an antibody.

As used herein, a "derivatized" antibody is one that has been

modified. Methods of derivatization include but are not limited to the addition of a fluorescent moiety, a radionuclide, a toxin, an enzyme or an affinity ligand such as biotin.

As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. In one embodiment of the humanized forms of the antibodies, some, most or all of the amino acids outside the CDR regions have been replaced with amino acids from human immunoglobulin molecules but where some, most or all amino acids within one or more CDR regions are unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they would not abrogate the ability of the antibody to bind a given antigen. Suitable human immunoglobulin molecules would include IgG1, IgG2, IgG3, IgG4, IgA, IgE and IgM molecules. A "humanized" antibody would retain a similar antigenic specificity as the original antibody. One skilled in the art would know how to make the humanized antibodies of the subject invention. Various publications, several of which are hereby incorporated by reference into this application, also describe how to make humanized antibodies. For example, the methods described in United States Patent No. 4,816,567 comprise the production of chimeric antibodies having a variable region of one antibody and a constant region of another antibody.

United States Patent No. 5,225,539 describes another approach for the production of a humanized antibody. This patent describes the use of recombinant DNA technology to produce a humanized antibody wherein the CDRs of a variable region of one immunoglobulin are replaced with the CDRs from an immunoglobulin with a different specificity such that the humanized antibody would recognize the desired target but would not be recognized in a significant way by the human subject=s immune system. Specifically, site directed

mutagenesis is used to graft the CDRs onto the framework.

Other approaches for humanizing an antibody are described in United States Patent Nos. 5,585,089 and 5,693,761 and WO 90/07861 which describe methods for producing humanized immunoglobulins. These have one or more CDRs and possible additional amino acids from a donor immunoglobulin and a framework region from an accepting human immunoglobulin. These patents describe a method to increase the affinity of an antibody for the desired antigen. Some amino acids in the framework are chosen to be the same as the amino acids at those positions in the donor rather than in the acceptor. Specifically, these patents describe the preparation of a humanized antibody that binds to a receptor by combining the CDRs of a mouse monoclonal antibody with human immunoglobulin framework and constant regions. Human framework regions can be chosen to maximize homology with the mouse sequence. A computer model can be used to identify amino acids in the framework region which are likely to interact with the CDRs or the specific antigen and then mouse amino acids can be used at these positions to create the humanized antibody.

The above patents 5,585,089 and 5,693,761, and WO 90/07861 also propose four possible criteria which may used in designing the humanized antibodies. The first proposal was that for an acceptor, use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. The second proposal was that if an amino acid in the framework of the human immunoglobulin is unusual and the donor amino acid at that position is typical for human sequences, then the donor amino acid rather than the acceptor may be selected. The third proposal was that in the positions immediately adjacent to the 3 CDRs in the humanized immunoglobulin chain, the donor amino acid rather than the acceptor amino acid may be selected.

The fourth proposal was to use the donor amino acid reside at the framework positions at which the amino acid is predicted to have a side chain atom within 3D of the CDRs in a three dimensional model of the antibody and is predicted to be capable of interacting with the CDRs. The above methods are merely illustrative of some of the methods that one skilled in the art could employ to make humanized antibodies.

In one embodiment of the above antibodies, the viral envelope protein is derived from HIV-1.

As used herein "derived" means obtained in whole or in part from HIV in the form of genomic sequences, primary isolates, molecular clones, consensus sequences and encompasses chimeras, and sequences modified by means such as truncations and point mutations.

This invention provides an isolated nucleic acid molecule encoding the above antibody. The nucleic acid molecule includes but is not limited to RNA, genomic DNA and cDNA.

This invention provides a method of reducing the likelihood of a virally exposed subject from becoming infected with the virus comprising administering the above antibody or the above isolated nucleic acid, thereby reducing the likelihood of the subject from becoming infected with the virus. In a preferred embodiment, the virus is HIV.

As used herein, "reducing the likelihood" means a smaller chance than would exist in a control situation without administration of the nucleic acid, protein or antibody.

This invention provides a method of treating a subject infected with a virus comprising administering the above antibody or the above isolated nucleic acid, thereby treating the subject. In a

preferred embodiment, the virus is HIV.

This invention provides an agent capable of binding the mutant viral envelope protein encoded by the above recombinant nucleic acid molecule. In one embodiment, the agent inhibits viral infection. In one embodiment, the viral envelope protein is derived from HIV-1.

As used herein, "agent" includes but is not limited to small organic molecules, antibodies, polypeptides, and polynucleotides.

As used herein, "inhibits viral infection" means reduces the amount of viral genetic information introduced into a target cell population as compared to the amount that would be introduced without said composition.

This invention provides a method for determining whether a compound is capable of inhibiting a viral infection comprising:
a. contacting an appropriate concentration of the compound with the mutant viral envelope protein encoded by the recombinant nucleic acid of claim 1 under conditions permitting binding of the compound to said protein;
b. contacting the resulting complex with a reporter molecule under conditions that permit binding of the reporter molecule to the mutant viral envelope protein;
c. measuring the amount of bound reporter molecule; and
d. comparing the amount of bound reporter molecule in step (C) with the amount determined in the absence of the compound, a decrease in the amount indicating that the compound is capable of inhibiting infection by the virus, thereby determining whether a compound is capable of inhibiting a viral infection.

Methods such as surface plasmon resonance may also be used to measure the direct binding of the compound to the mutant viral envelope protein using commercially available instruments,

methods and reagents (Biacore, Piscataway, N.J.).

As used herein "reporter molecule" means a molecule which when bound to mutant envelope proteins can be detected. Such molecules include but are not limited to radio-labeled or fluorescently-labeled molecules, enzyme-linked molecules, biotinylated molecules or similarly affinity tagged molecules, or molecules which are reactive with antibodies or other agents that are so labeled.

As used herein "measuring" can be done by any of the methods known to those skilled in the art. These include but are not limited to fluorometric, colorimetric, radiometric or surface plasmon resonance methods.

In one embodiment, the reporter molecule is an antibody or derivative thereof. In one embodiment, the virus is HIV-1. In one embodiment, the reporter molecule comprises one or more host cell viral receptors or molecular mimics thereof.

As used herein "molecular mimics" means a molecule with similar binding specificity.

This invention provides a method for determining whether a compound is capable of inhibiting a viral infection which comprises:

a. contacting an appropriate concentration of the compound with a host cell viral receptor or molecular mimic thereof under conditions that permit binding of the compound and receptor or receptor mimic;

b. contacting the resulting complex with the mutant viral envelope protein encoded by the recombinant nucleic acid of claim 1 under conditions that permit binding of the envelope protein and receptor or receptor mimic in the absence of the compound;

c. measuring the amount of binding of envelope protein to

receptor or receptor mimic;

d. comparing the amount of binding determined in step (c) with the amount determined in the absence of the compound, a decrease in the amount indicating that the compound is capable of inhibiting infection by the virus, thereby determining whether a compound is capable of inhibiting a viral infection.

In one embodiment of the above method, the virus is HIV-1. In one embodiment, the host cell viral receptor is CD4, CCR5, CXCR4 or combinations or molecular mimics thereof.

As used herein "CD4" means the mature, native, membrane-bound CD4 protein comprising a cytoplasmic domain, a hydrophobic transmembrane domain, and an extracellular domain which binds to the HIV-1 gp120 envelope glycoprotein. CD4 also comprises portions of the CD4 extracellular domain capable of binding to the HIV-1 gp120 envelope glycoprotein.

As used herein, "CCR5" is a chemokine receptor which binds members of the C-C group of chemokines and whose amino acid sequence comprises that provided in Genbank Accession Number 1705896 and related polymorphic variants. As used herein, CCR5 includes extracellular portions of CCR5 capable of binding the HIV-1 envelope protein.

As used herein, "CXCR4" is a chemokine receptor which binds members of the C-X-C group of chemokines and whose amino acid sequence comprises that provided in Genbank Accession Number 400654 and related polymorphic variants. As used herein, CXCR4 includes extracellular portions of CXCR4 capable of binding the HIV-1 envelope protein.

This invention provides a compound isolated using the above methods.

Pharmaceutically acceptable carriers are well known to those skilled in the art and include but are not limited to 0.01-0.1M and preferably 0.05M phosphate buffer, phosphate-buffered saline, or 0.9% saline. Additionally, such pharmaceutically acceptable carriers may include but are not limited to aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

This invention provides a compound determined to be capable of inhibiting a viral infection by the above methods.

This invention provides a pharmaceutical composition comprising an amount of the compound effective to inhibit viral infection determined by the above methods to be capable of inhibiting viral infection and a pharmaceutically acceptable carrier. In one embodiment, the viral infection is HIV infection. In the preferred embodiment, the viral infection is HIV-1 infection.

This invention provides a mutant complex comprising an immunodeficiency virus surface protein and an immunodeficiency virus transmembrane protein, wherein the mutant complex contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and transmembrane protein, compared to the stability of the wildtype complex. In one embodiment, the stability of the complex

is enhanced by introducing at least one disulfide bond between the transmembrane protein and the surface protein. In one embodiment, an amino acid residue in the transmembrane protein is mutated to a cysteine residue, resulting in the formation of a disulfide bond between the transmembrane protein and surface protein. In one embodiment, an amino acid residue in the surface protein protein is mutated to a cysteine residue, resulting in the formation of a disulfide bond between the transmembrane protein and surface protein. In one embodiment an amino acid residue in the transmembrane protein is mutated to a cysteine residue, and an amino acid residue in the surface protein protein is mutated to a cysteine residue, resulting resulting in the formation of a disulfide bond between the transmembrane protein and surface protein.

In one embodiment, immunodeficienecy virus is a human imunodeficiency virus. The human imunodeficiency virus includes but is not limited to the JR-FL strain. The surface protein includes but is not limited to gp120. An amino acid residue of the C1 region of gp120 may be mutated. An amino acid residue of the C5 region of gp120 may be mutated. The amino acids residues which may be mutated include but are not limited to the following amino acid residues: V35; Y39, W44; G462; I482; P484; G486; A488; P489; A492; and E500. The gp120 amino acid residues are also set forth in Figure 3A. The transmembrane protein includes but is not limited to gp41. An amino acid in the ectodomain of gp41 may be mutated. The amino acids residues which may be mutated include but are not limited to the following amino acid residues: D580; W587; T596; V599; and P600. The gp41 amino acid residues are also set forth in Figure 3B.

This invention provides a mutant viral envelope protein which differs from the corresponding wild type protein in at least one amino acid which yields a complex comprising a surface protein and a transmembrane protein which has enhanced stability relative

to the corresponding complex obtained from the wild type envelope protein, wherein the surface protein and transmembrane protein are encoded by different nucleic acids.

This invention provides a complex comprising a viral surface protein and a viral transmembrane protein which has enhanced stability relative to the corresponding complex obtained from the wildtype envelope protein, yielded by the proteolysis of a mutant viral envelope protein with a sequence which differs from the corresponding wild type protein sequence in at least one amino acid, wherein the surface protein and transmembrane protein are encoded by different nucleic acids.

This invention provides a nucleic acid which encodes a mutant surface protein wherein the surface protein is complexed with its corresponding transmembrane protein and will have enhanced stability.

This invention provides a nucleic acid which encodes a mutant transmembrane protein wherein the transmembrane protein is complexed with its corresponding surface protein and will have enhanced stability.

This invention provides an antibody which binds to the above protein or above complex but does not cross react with the individual monomeric surface protein or the individual monomeric transmembrane protein.

This invention provides the above antibody capable of binding to the virus.

This invention provides a protein comprising at least a portion of a viral envelope protein which differs from the corresponding wild type protein in at least one amino acid which yields a complex comprising a surface protein and a transmembrane protein

which has enhanced stability relative to the corresponding complex obtained from the wild type envelope protein, wherein the portion of the protein results in enhanced stability.

This invention provides a portion of the above protein, wherein the portion results in enhanced immunogenicity in comparison to the corresponding wild type portion.

This invention further provides a simple method for determining whether a subject has produced antibodies capable of blocking the infectivity of a virus. This diagnostic test comprises examining the ability of the antibodies to bind to the stabilized viral envelope protein. As shown herein, such binding is indicative of the antibodies' ability to neutralize the virus. In contrast, binding of antibodies to non-stabilized, monomeric forms of viral envelope proteins is not predictive of the antibodies' ability to bind and block the infectivity of infectious virus (Fouts et al., J. Virol. 71:2779, 1997). The method offers the practical advantage of circumventing the need to use infectious virus.

Numerous immunoassay formats that are known to the skilled artisan are appropriate for this diagnostic application. For example, an enzyme-linked immunosorbent assay (ELISA) format could be used wherein in the mutant virus envelope glycoprotein is directly or biospecifically captured onto the well of a microtiter plate. After wash and/or blocking steps as needed, test samples are added to the plate in a range of concentrations. The antibodies can be added in a variety of forms, including but not limited to serum, plasma, and a purified immunoglobulin fraction. Following suitable incubation and wash steps, bound antibodies can be detected, such as by the addition of an enzyme-linked reporter antibody that is specific for the subject's antibodies. Suitable enzymes include horse radish peroxidase and alkaline phosphatase, for which numerous immunoconjugates and colorimetric substrates are commercially available. The binding

of the test antibodies can be compared with that of a known monoclonal or polyclonal antibody standard assayed in parallel. In this example, high level antibody binding would indicate high neutralizing activity.

As an example, the diagnostic test could be used to determine if a vaccine elicited a protective antibody response in a subject, the presence of a protective response indicating that the subject was successfully immunized and the lack of such response suggesting that further immunizations are necessary. In a preferred embodiment, the subject is a human.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Part I

Materials and Methods

1. Materials

The plasmid designated PPI4-tPA-gp120$_{JR-FL}$ was deposited pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 under ATCC Accession Nos. 75431. The plasmid was deposited with ATCC on March 12, 1993. This eukaryotic shuttle vector contains the cytomegalovirus major immediate-early (CMV MIE) promoter/enhancer linked to the full-length HIV-1 envelope gene whose signal sequence was replaced with that derived from tissue plasminogen activator. In the vector, a stop codon has been placed at the gp120 C-terminus to prevent translation of gp41 sequences, which are present in the vector. The vector also contains an ampicillin resistance gene, an SV40 origin of replication and a DHFR gene whose transcription is driven by the â-globin promoter.

The epitopes for, and some immunochemical properties of, anti-gp120 Mabs from various donors have been described previously (Moore et al., J. Virol. 768: 469, 1994; Moore and Sodroski, J. Virol. 70:1863, 1996). These include Mab 19b to the V3 locus (Moore et al., J. Virol. 69:122, 1995); mABs 50.1 and 83.1 to the V3 loop (White-Scharf et al. Virology 192:197, 1993); MAbs IgG1b12 and F91 to the CD4 binding site (CD4bs) (Burton et al., Science 266: 124, 1994; Moore and Sodroski, J. Virol. 70:1863, 1996) Mab 2G12 to a unique C3-V4 glycan-dependent epitope (Trkola et al., J. Virol. 70:1100, 1996) MAb M90 to the C1 region (diMarzo Veronese et al. AIDS Res. Human Retrov. 8:1125, 1992); Mab 23a and Ab D7324 to the C5 region (Moore and Sodroski, J. Virol. 70:1863, 1996); Mab 212A to a conformational C1-C5 epitope

(Moore et al. J. Virol 68:6836, 1994); Mab 17b to a CD4-inducible epitope (Moore and Sodroski, J. Virol. 70:1863, 1996); Mab A32 to a CD4-inducible C1-C4 epitope (Moore and Sodroski, J. Virol. 70:1863, 1996; Sullivan et al, J. Virol. 72:4694, 1998); Mabs G3-519 and G3-299 to C4 or C4/V3 epitopes (Moore and Sodroski, J. Virol. 70:1863, 1996). Mabs to gp41 epitopes included 7B2 to epitope cluster 1(kindly provided by Jim Robinson, Tulane University); 25C2 to the fusion peptide region (Buchacher et al. AIDS Res. Human Retrov. 10:359, 1994); 2F5 to a neutralizing epitope encompassing residues 665-690 (Munster et al. J. Virol. 68:4031, 1994). The tetrameric CD4-IgG2 has been described previously (Allaway et al. AIDS Res. Human Retrovir. 11:533, 1995).

Anti-HIV Abs were obtained from commercial sources, from the NIH AIDS Reagent Program, or from the inventor. Where indicated, the Abs were biotinylated with NHS-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions.

Monomeric $gp120_{JR-FL}$ was produced in CHO cells stably transfected with the PPI4-tPA-$gp120_{JR-FL}$ plasmid as described (U.S. Patents 5,866,163 and 5,869,624). Soluble CD4 was purchased from Bartels Corporation (Issaquah, WA).

2. Construction of PPI4-based plasmids expressing wild-type and mutant HIV envelope proteins

Wild-type gp140s (gp140WT) The gp140 coding sequences were amplified using the polymerase chain reaction (PCR) from full-length molecular clones of the HIV-1 isolates JR-FL, DH123, Gun-1, 89.6, NL4-3 and HxB2. The 5' primer used was designated Kpn1env (5'-GTCTATTATGGGGTACCTGTGTGGAA AGAAGC-3') while the 3' primer was BstB1env (5=-CGCAGACGCAGATTCGAATTAATACCACAGCCAGTT-3=). PCR was performed under stringent conditions to limit the extent of *Taq* polymerase-introduced error. The PCR products were

digested with the restriction enzymes *Kpn*1 and *Xho*1 and purified by agarose gel electrophoresis. Plasmid PPI4-tPA-gp120$_{JR-FL}$ was also digested with the two restriction enzymes and the large fragment (vector) was similarly gel-purified. The PPI4-tPA-gp120$_{JR-FL}$ expression vector has been described previously (Hasel and Maddon, U.S. Patents #5886163 and 5869624). Ligations of insert and vector were carried out overnight at room temperature. DH5áF=Q10 bacteria were transformed with 1/20 of each ligation. Colonies were screened directly by PCR to determine if they were transformed with vector containing the insert. DNA from three positive clones of each construct were purified using a plasmid preparation kit (Qiagen, Valencia, CA) and both strands of the entire gp160 were sequenced. By way of example, pPPI4-gp140WT$_{JR-FL}$ and pPPI4-gp140WT$_{DH123}$ refer to vectors expressing wild-type, cleavable gp140s derived from HIV-1$_{JR-FL}$ and HIV-1$_{DH123}$, respectively.

gp140UNC A gp120-gp41 cleavage site mutant of JR-FL gp140 was generated by substitutions within the REKR motif at the gp120 C-terminus, as described previously (Earl et al., Proc. Natl. Acad. Sci. USA 87:648, 1990). The deletions were made by site-directed mutagenesis using the mutagenic primers 5'140M (5=-CTACGACTTCGTCTCCGCCTT CGACTACGGGGAATAGGAGCTGTGTTCCTTGGGTTCTTG-3=) and 3'gp140M (sequence conjunction with Kpn1env and BstB1env 5'-TCGAAGGCGGAGACGAAGTCGTAGCCGCAGTGCCTTGGTGG GTGCTACTCCTAATGGTTC-3'). In conjunction with Kpn1env and BstB1, the PCR product was digested with *Kpn*1 and *Bst*B1 and subcloned into pPPI4 as described above.

Loop-deleted gp120s and gp140s PPI4-based plasmids expressing variable loop-deleted forms of gp120 and gp140 proteins were prepared using the splicing by overlap extension method as described previously (Binley *et al.*, AIDS Res. Human Retrovir. 14:191, 1998). In the singly loop-deleted mutants, a Gly-Ala-Gly spacer is used to replace D132-K152 (ΔV1), F156-I191 (ΔV2), or

T300-G320 (ΔV3). The numbering system corresponds to that for the JR-FL clone of HIV-1 (Genbank Accession # U63632).

PCR amplification using DGKPN5'PPI4 and 5JV1V2-B (5'-GTCTATTATGGGGTACCTGTGTGGAAAGAAGC-3') on a ΔV1 template and subsequent digestion by *Kpn*1 and *Bam*H1 generated a 292bp fragment lacking the sequences encoding the V1 loop. This fragment was cloned into a plasmid lacking the sequences for the V2 loop using the Kpn1 and BamH1 restriction sites. The resulting plasmid was designated ΔV1V2' and contained a Gly-Ala-Gly sequences in place of both D132-K152 and F156-I191. Envs lacking the V1, V2 and V3 loops were generated in a similar way using a fragment generated by PCR on a ΔV3 template with primers 3JV2-B (5'-GTCTGAGTCGGATCCTGTGACACCTCAGTCATTACACAG-3') and H6NEW (5'CTCGAGTCTTCGAATTAGTGATGGGTGATGGTGATGATACCACAGCCATTTTGTTATGTC-3'). The fragment was cloned into ΔV1V2', using BamH1 and BstB1. The resulting env construct was named ΔV1V2'V3. The glycoproteins encoded by the ΔV1V2' and ΔV1V2'V3 plasmids encode a short sequence of amino acids spanning C125 to C130. These sequences were removed using mutagenic primers that replace T127-I191 with a Gly-Ala-Gly sequence. We performed PCR amplification with primers 3'DV1V2STU1 (5'-GGCTCAAAGGATATCTTTGGACAGGCCTGT GTAATGACTGAGGTGTCACATCCTGCACCACAGAGTGGGGTTAATTTTACACATGGC-3') and DGKPN5'PPI4, digested the resulting fragment by *Stu*1 and *Kpn*1 and cloned it in a PPI4 gp140 vector. The resulting gp140 was named ΔV1V2*. In an analogous manner ΔV1V2*V3 was constructed. The amino acid substitutions are shown schematically in Figure 10.

Glycosylation site mutants Canonical N-linked glycosylation sites were eliminated at positions 357 and 398 on gp120 by point mutations of asparagine to glutamine. These changes were made on templates encoding both wild-type and loop-deleted HIV envelope proteins.

Disulfide-stabilized gp140s The indicated amino acids in gp120

and gp41 were mutated in pairs to cysteines by site-directed mutagenesis using the Quickchange kit (Stratagene, La Jolla, CA). As indicated below, additional amino acids in the vicinity of the introduced cysteines were mutated to alanines using similar methods in an attempt to better accommodate the cysteine mutations within the local topology of the envelope glycoproteins. The changes were similarly made on templates encoding both wild-type and loop-deleted HIV envelope proteins.

3. Expression of gp140s in transiently transfected 293T cells

HIV envelope proteins were transiently expressed in adherent 293T cells, a human embryonic kidney cell line (ATCC Cat. # CRL-1573) transfected with the SV40 large T antigen, which promotes high level replication of plasmids such as PPI4 that contain the SV40 origin. 293T cells were grown in Dulbecco's minimum essential medium (DMEM; Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum supplemented with L-glutamine, penicillin, and streptomycin. Cells were plated in a 10 cm dish and transfected with 10 μg of purified PPI4 plasmid using the calcium phosphate precipitation method. On the following day, cells were supplied fresh DMEM containing 0.2% bovine serum albumin along with L-glutamine, penicillin and streptomycin. For radioimmunoprecipitation assays, the medium also contained ^{35}S-labeled cysteine and methionine (200 μCi/ plate). In certain experiments, the cells were cotransfected with 10 μg of a pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) encoding the gene for human furin.

4. ELISA analyses

The concentration of gp120 and gp140 proteins in 293T cell supernatants was measured by ELISA (Binley et al. J. Virol 71:2799, 1997). Briefly, Immulon II ELISA plates (Dynatech Laboratories, Inc.) were coated for 16-20 hr at 4 EC with a polyclonal sheep antibody that recognizes the carboxy-terminal sequence of gp120 (APTKAKRRVVQREKR). The plate was washed with

tris buffered saline (TBS) and then blocked with 2% nonfat milk in TBS. Cell supernatants (100 μL) were added in a range of dilutions in tris buffered saline containing 10% fetal bovine serum. The plate was incubated for 1 hr at ambient temperature and washed with TBS. Anti-gp120 or anti-gp41 antibody was then added for an additional hour. The plate was washed with TBS, and the amount of bound antibody is detected using alkaline phosphatase conjugated goat anti-human IgG or goat anti-mouse IgG. Alternatively, biotinylated reporter Abs are used according to the same procedure and detected using a streptavidin-AP conjugate. In either case, AP activity is measured using the AMPAK kit (DAKO) according to the manufacturer's instructions. To examine the reactivity of denatured HIV envelope proteins, the cell supernatants were boiled for 5 minutes in the presence of 1% of the detergents sodium dodecyl sulfate and NP-40 prior to loading onto ELISA plates in a range of dilutions. Purified recombinant JR-FL gp120 was used as a reference standard.

5. Radioimmunoprecipitation assay (RIPA)

^{35}S-labeled 293T cell supernatants were collected 2 days post-transfection for RIPA analysis. Culture supernatants were cleared of debris by low speed centrifugation (~ 300g) before addition of RIPA buffer to a final concentration of 50 mM tris-HCl, 150mM NaCl, 5 mM EDTA, pH 7.2. Biotinylated Abs (~10 μg) were added to 1 mL of supernatant and incubated at ambient temperature for 10 min. Samples were then incubated with streptavidin-agarose beads for 12-18hr at 4 EC with gentle agitation. Alternatively, unlabeled Abs were used in combination with protein G-agarose (Pierce, Rockford, IL). The beads were washed three times with RIPA buffer containing 1% Nonidet-P40 (NP40) detergent. Bound proteins were eluted by heating at 100 EC for 5 min with SDS-PAGE sample buffer containing 0.05 M tris-HCl, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.001% bromophenol blue, and where indicated, 100mM dithiothreitol (DTT). Samples were loaded on an 8% polyacrylamide gel and run at 200V for 1 hour. Gels were

then dried and exposed to a phosphor screen for subsequent image analysis using a STORM phosphoimager (Molecular Dynamics, Sunnyvale, CA). ^{14}C-labeled proteins were used as size calibration standards (Life Technologies, Gaithersburg, MD).

Experimental results

1. Processing of gp140NON is facilitated by co-expression of the furin protease

To minimize the production of gp140NON, pcDNA3.1-furin and pPPI4-$gp140WT_{JR-FL}$ were cotransfected into 293T cells, and RIPA assay was performed using the anti-gp120 MAb 2G12. As indicated in Figure 2, furin eliminated production of gp140NON but had no effect on gp140UNC. Similar results were obtained in RIPAs performed using other anti-gp120 MAbs .

Treatment of the samples with DTT prior to SDS-PAGE did not affect the migration or relative amounts of these bands, indicating that the gp140s consist of a single polypeptide chain rather than separate gp120-gp41 molecules linked by an adventitious disulfide bond.

2. Stabilization of the gp120-gp41 interaction by introduction of double cysteine mutations

With furin co-transfection, we could now express a soluble gp140 protein in which the gp120 and $gp41_{ECTO}$ components were associated only through a non-covalent linkage, mimicking what occurs in the native trimeric envelope glycoprotein complex on virions. However, on virions or the surface of infected cells, the gp120-gp41 association is weak, so that gp120 is gradually shed (McKeating et al. J. Virol 65:852, 1991). We found this to occur also with the gp140WT protein made in the presence of endogenous furin. Thus, we could detect very little, if any, stable gp120-gp41ECTO complexes in the supernatants from gp140WT-expressing cells after immunoprecipitation. We therefore sought ways to stabilize the non-covalent gp120-gp41 interaction, by the introduction of an intermolecular disulfide bond between the gp120 and gp41 subunits.

We therefore substituted a cysteine residue at one of several

different positions in the C1 and C5 regions of gp120, focussing on amino acids previously shown to be important for the gp120-gp41 interaction (Fig.3a). Simultaneously, we introduced a second cysteine mutation at several residues near the intramolecular disulfide loop of gp41 (Fig.3b). The intent was to identify pairs of cysteine residues whose physical juxtaposition in native gp120-gp41 was such that an intermolecular disulfide bond would form spontaneously. In all, >50 different double-cysteine substitution mutants were generated in the context of the JR-FL gp140WT protein, and co-expressed with furin in transient transfections of 293T cells .

An initial analysis of the transfection supernatants by antigen capture ELISA indicated that all of the mutants were efficiently expressed as secreted proteins, except those which contained a cysteine at residue 486 of gp120 . We next characterized the transfection supernatants by immunoprecipitation with the anti-gp120 MAbs 2G12 and F91 (Fig.4). In addition to the expected 120kDa band (gp120), a second band of approximately 140kDa was precipitated by F91 and 2G12 from many of the double-cysteine mutant transfection supernatants. The gp140 bands derived from mutants in which a cysteine was present in the C1 region of gp120 migrated slightly more slowly, and were more diffuse, than the corresponding bands from mutants in which the gp120 cysteine was in the C5 region (Fig.4). The presence of diffuse bands with reduced mobility on SDS-PAGE gels is probably indicative of incomplete or improper envelope glycoprotein processing, based on previous reports (Earl et al. Proc. Natl. Acad. Sci. USA 87:648, 1990; Earl et al. J. Virol 68:3015, 1994). The relative intensity of the 140kDa band was highly dependent upon the positions of the introduced cysteines, suggesting that certain steric requirements must be met if a stable intersubunit disulfide bond is to be formed.

To determine which among the double-cysteine mutants was the most

suitable for further analysis, we determined the relative intensities of the gp140 and gp120 bands derived after immunoprecipitation of each mutant by the potently neutralizing anti-gp120 MAb 2G12, followed by SDS-PAGE and densitometry (Figure 5). We sought the mutant for which the gp140/gp120 ratio was the highest, which we interpreted as indicative of the most efficient formation of the intermolecular disulfide bond. From Figure 5, it is clear that mutant A492C/T596C has this property. From hereon, we will refer to this protein as the SOS gp140 mutant. Of note is that the mobility of the SOS gp140 mutant on SDS-PAGE is identical to that of the $gp140_{NON}$ protein, in which the gp120 and $gp41_{ECTO}$ moieties are linked by a peptide bond. The gp140 band derived from the SOS mutant is not quite as sharp as that from the $gp140_{NON}$ protein, but it is less diffuse than the gp140 bands obtained from any of the other double-cysteine mutants (Fig. 4). This suggests that the SOS mutant is efficiently processed. The complete nucleic acid and amino acid sequences of the JR-FL SOS gp140 mutant are provided in Figure 13.

We verified that the 140kDa proteins were stabilized by an intermolecular disulfide bond by treating the immunoprecipitated proteins with DTT prior to gel electrophoresis. In contrast, the 140 kDa bands in $gp14_{0WT}$ and $gp140_{UNC}$ were unaffected by the DTT treatment as expected for uncleaved single-chain proteins. Of note is that a 140kDa band was never observed for either the A492C or T596C single mutants.(Fig.6b). This is further evidence that the 140kDa band in the double-cysteine mutants arises from the formation of an intermolecular disulfide bond between gp120 and gp41ECTO. In the absence of exogenous furin, the 140kDa SOS protein band was not reducible by DTT, suggesting the band is the double cysteine mutant of $gp140_{NON}$ (Fig. 6C).

3. Approaches to improve the efficiency of disulfide bond formation in the SOS gp140 protein

Disulfide-stabilized gp140 is not the only env species present in the 293T cell supernatants. Discernable amounts of free gp120 are also present. This implies that the disulfide bond between gp120 and the gp41 ectodomain forms with imperfect efficiency. Although the free gp120 can be removed by the purification methods described below, attempts were made to further reduce or eliminate its production. To this end, additional amino acid substitutions were made near the inserted cysteines. In addition, the position of the cysteine in gp120 was varied. We retained the gp41 cysteine at residue 596, as in the SOS gp140 protein, because this position seemed to be the one at which intermolecular disulfide bond formation was most favored.

We first varied the position of the cysteine substitution in gp120, by placing it either N-terminal or C-terminal to alanine-492. The gp140/gp140+gp120 ratio was not increased in any of these new mutants; it remained comparable with, or less than, the ratio derived from the SOS gp140 protein (Fig.7). Furthermore, there was usually a decrease in the mobility and sharpness of the gp140 band compared to that derived from the SOS gp140 protein (Fig.7). Next, we considered whether the bulky side chains of the lysine residues adjacent to alanine-492 might interfere with disulfide bond formation. We therefore mutated the lysines at positions 491 and 493 to alanines in the context of the SOS gp140 protein, but these changes neither increased the gp140/gp140+gp120 ratio nor affected the migration of gp140 (Fig.7). Finally, we introduced a second pair of cysteines into the SOS gp140 protein at residues 44 of gp120 and 600 of gp41, since a disulfide bond formed fairly efficiently when this cysteine pair was introduced into the wild-type protein (Fig.5). However, the quadruple-cysteine mutant (W44C/A492C/P600C/T596C) was poorly expressed, implying that there was a processing or folding problem (Fig.7). Poor expression was also observed with two more quadruple-cysteine mutants (W44C/K491C/P600C/T596C and (W44C/K493C/P600C/T596C) (Fig.7).

Further approaches to optimize the efficiency or overall expression of the disulfide stabilized mutant are possible. For example, cells stably transfected with furin could be created so as to ensure adequate levels of furin in all cells expressing the SOS gp140 proteins. Similiarly, furin and the gp140 proteins could be coexpressed from a single plasmid. K491 and K493 could be mutated to non-alanine residues singly or as a pair. To better accomodate the introduced cysteines, other gp120 and/or gp41 amino acids in the vicinity of the introduced cysteines could be mutated as well.

4. The antigenicity of the SOS gp140 protein parallels that of virus-associated gp120-gp41

Compared to $gp140_{NON}$, the SOS gp140 protein has several antigenic differences that we believe are desirable for a protein intended to mimic the structure of the virion-associated gp120-gp41 complex. These are summarized below.

1) The SOS gp140 protein binds strongly to the potently neutralizing MAbs IgG1b12 and 2G12, and also to the CD4-IgG2 molecule (Fig.8a). Although the RIPA methodology is not sufficiently quantitative to allow a precise determination of relative affinities, the reactivities of these MAbs and of the CD4-IgG2 molecule with the SOS gp140 protein appear to be substantially greater than with the $gp140_{NON}$ and gp120 proteins (Fig.8a). Clearly, the SOS gp140 protein has an intact CD4-binding site. V3 loop epitopes are also accessible on the SOS gp140 protein, shown by its reactivity with MAbs 19b and 83.1 (Fig. 8a).

2) Conversely, several non-neutralizing anti-gp120 MAbs bind poorly, or not at all, to the SOS gp140 protein whereas they react strongly with $gp140_{NON}$ and gp120 (Fig.8b). These MAbs include ones directed to the C1 and C5 domains, regions of gp120 that are involved in gp41 association and which are considered

to be occluded in the context of a properly formed gp120-gp41 complex (Moore et al. J. Virol 68:469, 1994; Wyatt et al. J. Virol. 71:9722, 1997). Conversely, the C1- and C5-directed MAbs all reacted strongly with the $gp140_{NON}$ protein (Fig.8b).

3) The exposure of the epitope for MAb 17b by the prior binding of soluble CD4 occurs far more efficiently on the SOS gp140 protein than on the $gp140_{NON}$ or gp120 proteins (Fig.8c). Indeed, in the absence of soluble CD4, there was very little reactivity of 17b with the SOS gp140 protein. The CD4-induced epitope for MAb 17b overlaps the coreceptor binding site on gp120; it is considered that this site becomes exposed on the virion-associated gp120-gp41 complex during the conformational changes which initiate virus-cell fusion after CD4 binding. Induction of the 17b epitope suggests that the gp120 moieties on the SOS gp140 protein possess the same static conformation and conformational freedom as virus-associated gp120-gp41. The $gp140_{NON}$ protein bound 17b constitutively, and although there was some induction of the 17b epitope upon soluble CD4 binding, this was less than occurred with the SOS gp140 protein.

4) Another CD4-inducible epitope on gp120 is that recognized by MAb A32 (Moore et al. J. Virol. 70:1863, 1996; Sullivan et al. J. Virol. 72:4694, 1998). There was negligible binding of A32 to the SOS gp140 mutant in the absence of soluble CD4, but the epitope was strongly induced by soluble CD4 binding (Fig. 8c). As observed with 17b, the A32 epitope was less efficiently induced on the $gp140_{NON}$ protein than on the SOS gp140 protein.

5) There was no reactivity of any of a set of non-neutralizing gp41 MAbs with the SOS gp140 protein, whereas all of these MAbs bound strongly to the $gp140_{NON}$ protein. These anti-gp41 MAbs recognize several regions of the gp41 ectodomain, all of which are thought to be occluded by gp120 in the virion-associated gp120-gp41 complex (Moore et al. J. Virol. 68:469, 1994; Sattentau et al. Virology 206:713, 1995). Their failure to

bind to the SOS gp140 protein is another strong indication that this protein adopts a configuration similar to that of the native trimer; their strong recognition of the $gp140_{NON}$ protein is consistent with the view that these proteins have an aberrant conformation because of the peptide bond linking gp120 with gp41 (Edinger et al. J. Virol. 73:4062, 1999)(Fig. 8d).

6) In marked contrast to what was observed with the non-neutralizing MAbs, the neutralizing anti-gp41 MAb 2F5 bound efficiently to the SOS gp140 protein, but not to the $gp140_{NON}$ protein. Of note is that the 2F5 epitope is the only region of gp41 thought to be well exposed in the context of native gp120-gp41 complexes (Sattentau et al. Virology 206: 713, 1995). Its ability to bind 2F5 is again consistent with the adoption by the SOS gp140 protein of a configuration similar to that of the native trimer.

The antigenic properties of the SOS gp140 protein were compared with those of the W44C/T596C gp140 mutant. Among the set of mutants that contained a cysteine substitution within the C1 domain, this was the most efficient at gp140 formation. Although the W44C/T596C gp140 reacted well with the 2G12 MAb, it bound CD4-IgG2 and IgG1b12 relatively poorly. Furthermore, there was little induction of the 17b epitope on the W44C/T596C gp140 by soluble CD4, yet strong reactivity with non-neutralizing anti-gp41 MAbs (Fig.8). We therefore judge that this mutant has suboptimal antigenic properties. Indeed, the contrast between the properties of the W44C/T596C gp140 protein and the SOS gp140 protein demonstrates that the positioning of the intermolecular disulfide bonds has a significant influence on the antigenic structure of the resulting gp140 molecule.

In contrast to the antigenic character of the gp140SOS protein, the 140kDa proteins of $gp140_{WT}$ and $gp140_{UNC}$ reacted strongly with non-neutralizing anti-gp120 and anti-gp41 MAbs such as G3-519 and

7B2. In addition, the epitope recognized by MAb 17B was constitutively exposed rather than CD4-inducible (Fig. 8e). Overall, there was a strong correlation between the binding of MAbs to the SOS gp140 protein and their ability to neutralize HIV-1_{JR-FL}. This correlation was not observed with the gp140_{NON}, gp140_{UNC} or gp120 proteins.

5. The formation of intersubunit disulfide bonds is not isolate-dependent

To assess the generality of our observations with gp140 proteins derived from the R5 HIV-1 isolate JR-FL, we generated double-cysteine mutants of gp140=s from other HIV-1 strains. These include the R5X4 virus DH123 and the X4 virus HxB2. In each case, the cysteines were introduced at the residues equivalent to alanine-492 and threonine-596 of JR-FL. The resulting SOS proteins were transiently expressed in 293T cells and analyzed by RIPA to ascertain their assembly, processing and antigenticity. As indicated in Fig. 9, 140 kDa material is formed efficiently in the DH123 and HxB2 SOS proteins, demonstrating that our methods can successfully stabilize the envelope proteins of diverse viral isolates.

6. Disulfide stabilization of HIV envelope proteins modified in variable loop and glycosylation site regions

Since there is evidence to suggest that certain variable loop and glycosylation site mutations provide a means to better expose underlying conserved neutralization epitopes, we examined the assembly and antigenicity of disulfide-stabilized forms. In initial studies, A492C/T596C JR-FL gp140 mutants were created for each of the ΔV1, ΔV2, ΔV3, ΔV1V1*, and ΔV1V2*V3 molecules described above. For the ΔV1V2*V3 protein, glycosylation site mutants were also synthesized by N6Q point mutations of amino acids 357 and 398.

For each of the singly and doubly loop-deleted mutants, we could

detect gp140 bands in comparable quantities as for the full-length SOS gp140 protein (Fig. 11B). To see whether deletion of the variable loops altered antigenicity in an oligomeric context, we precipitated the ΔV3 and Δ1V2* SOS proteins with a panel of MAbs (Fig. 12). MAbs to gp41 except 2F5 did not bind to loop deleted versions of the cysteine stabilized protein, indicating that those epitopes are still occluded. MAbs to C1 and C5 epitopes were similarly non-reactive. The neutralizing antibody 2F5 did bind to the mutants and was particularly reactive with the ΔV3 SOS protein. MAbs to the CD4BS (IgG1b12, F91) as well as 2G12 bound avidly to these mutants as well. Of note is that CD4-IgG2 and 2G12 bound with very high affinity to the oligomeric ΔV3 SOS protein. Furthermore, consistent with data indicating that the CD4i epitopes are constitutively exposed on the ΔV1V2* protein, binding of MAbs 17b and A32 to the ΔV1V2* SOS mutant was not inducible by sCD4. The ΔV3 SOS mutant, however, bound 17b and A32 weakly in the absence of sCD4 and strongly in its presence. These results are consistent with observations that the V1/V2 and V3 loop structures are involved in occlusion of the CD4i epitopes (Wyatt *et al.*, J. Virol. 69:5723, 1995). Taken together, the results demonstrate that variable loop-deleted gp140s can be disulfide-stabilized without loss of conformational integrity. Figures 14 and 15, respectively, contain the complete nucleic acid and amino acid sequences of the ΔV1V2* and ΔV3 JR-FL SOS proteins.

For the ΔV1V2*V3 and ΔV1V2*V3 N357Q N398Q SOS mutants, we could not precipitate a gp140 (110 kDa and 105 kDa) with any of a variety of neutralizing and non-neutralizing MAbs (Fig. 11A, lanes 3, 4, 7 & 8). We did, however, observe strong 90 kDa and 85 kDa bands, which correspond to the mutant gp120 domains. These preliminary experiments suggest a variety of approaches for disulfide-stabilizing triply-loop deleted gp140s, including adjusting the location(s) of one or more introduced cysteines, adding additional pairs of cysteines, modifying amino acids

adjacent to the introduced cysteines, and modifying the manner in which the loops are deleted. Alternatively, triply loop deleted gp140s derived from other HIV isolates may be more readily stabilized by cysteines introduced at residues homologous to 496/592.

7. Production and purification of recombinant HIV-1 envelope glycoproteins

Milligram quantities of high quality HIV-1 envelope glycoproteins are produced in CHO cells stably transfected with PPI4 envelope-expressing plasmids (U.S. Patent 5,886,163 and 5,869,624). The PPI4 expression vector contains the dhfr gene under the control of the β-globin promoter. Selection in nucleoside-free media of dhfr$^-$ clones is followed by gene amplification using stepwise increases in methotrexate concentrations. The cytomegalovirus (CMV) promoter drives high level expression of the heterologous gene, and the tissue plasminogen activator signal sequence ensures efficient protein secretion. A high level of gp120 expression and secretion is obtained only upon inclusion of the complete 5= non-coding sequences of the CMV MIE gene up to and including the initiating ATG codon. To produce milligram quantities of protein, recombinant CHO cells are seeded into roller bottles in selective media and grown to confluency. Reduced serum-containing media is then used for the production phase, when supernatants are harvested twice weekly. A purification process comprising lectin affinity, ion exchange, and/or gel filtration chromatographies is carried out under non-denaturing conditions.

8. A protocol for determining the immunogenicity of stabilized HIV-1 envelope subunit proteins

Purified recombinant HIV-1 envelope proteins are formulated in suitable adjuvants (*e.g.*, Alum or Ribi Detox). For alum, formulation is achieved by combining the mutant HIV-1 envelope glycoprotein (in phosphate buffered saline, normal saline or

similar vehicle) with preformed aluminum hydroxide gel (Pierce, Rockford, IL) at a final concentration of approximately 500 μg/mL aluminum. The antigen is allowed to adsorb onto the alum gel for two hours at room temperature.

Guinea pigs or other animals are immunized 5 times, at monthly intervals, with approximately 100 μg of formulated antigen, by subcutaneous intramuscular or intraperitoneal routes. Sera from immunized animals are collected at biweekly intervals and tested for reactivity with HIV-1 envelope proteins in ELISA as described above and for neutralizing activity in well established HIV-1 infectivity assays (Trkola et al J. Virol 72: 1876, 1998). Vaccine candidates that elicit the highest levels of HIV-1 neutralizing Abs can be tested for immunogenicity and efficacy in preventing or treating infection in SHIV-macaque or other non-human primate models of HIV infection, as described below. The subunit vaccines could be used alone or in combination with other vaccine components, such as those designed to elicit a protective cellular immune response.

For these studies, the HIV-1 envelope proteins also may be administered in complex with one or more cellular HIV receptors, such as CD4, CCR5, and CXCR4. As described above, the binding of soluble CD4 exposes formerly cryptic conserved neutralization epitopes on the stabilized HIV-1 envelope protein. Antibodies raised to these or other neoepitopes could possess significant antiviral activity. As described above, interaction of CD4-env complexes with fusion coreceptors such as CCR5 and CXCR4 is thought to trigger additional conformational changes in env required for HIV fusion. Trivalent complexes comprising the stabilized env, CD4, and coreceptor could thus adopt additional fusion intermediary conformations, some of which are thought to be sufficiently long-lived for therapeutic and possibly immunologic interventions (Kilby et al. Nat. Med. 4:1302, 1998). Methods for preparing and administering env-CD4 and env-CD4-

coreceptor complexes are well-known to the skilled artisan (LaCasse et al., Science 283:357, 1999; Kang et al., J. Virol., 68:5854, 1994; Gershoni et al., FASEB J. 7:1185, 1993).

9. A protocol for determining the immunogenicity of nucleic acid-based vaccines encoding stabilized HIV-1 envelope proteins

PCR techniques are used to subclone the nucleic acid into a DNA vaccine plasmid vector such as pVAX1 available from Invitrogen (catalog #V260-20). PVAX1 was developed according to specifications in the FDA document, "Points to Consider on Plasmid DNA Vaccines for Preventive Infectious Disease Indications" published on December 22, 1996. PVAX1 has the following features: Eukaryotic DNA sequences are limited to those required for expression in order to minimize the possibility of chromosomal integration, Kanamycin is used to select the vector in E.coli because ampicillin has been reported to cause an allergic response in some individuals, Expression levels of recombinant proteins from pVAX1 is comparable to those achieved with its parent vector, pc DNA3.1, and the small size of pVAX1 and the variety of unique cloning sites amplify subcloning of even very large DNA fragments.

Several methods can be used to optimize expression of the disulfide stabilized protein *in vivo*. For example, standard PCR cloning techniques could be used to insert into pVAX1 certain elements of the optimized PPI4 expression vector, including Intron A and adjoining regions of the CMV promoter. In addition, the genomic DNA sequences of the HIV-1 envelope are biased towards codons that are suboptimal for expression in mammalian cells (Haas et al. Current Biol. 6:315, 1996). These can be changed to more favorable codons using standard mutagenesis techniques in order to improve the immunogenicity of nucleic acid based HIV vaccines (Andre et al., J. Virol. 72:1497, 1998). The codon optimization strategy could strive to increase the number of CpG motifs, which are known to increase the immunogencity of

DNA vaccines (Klinman et al., J. Immunol. 158:3635, 1997). Lastly, as for the transient transfection systems described above, env processing into gp120-gp41 may be facilitated by the heterologous expression of furin introduced on the same or separate expression vectors.

The insert containing plasmid can be administered to the animals by such means as direct injection or using gene gun techniques. Such methods are known to those skilled in the art.

In one protocol, Rhesus macaques are individually inoculated with five approximately 1mg doses of the nucleic acid. The doses are delivered at four week intervals. Each dose is administered intramuscularly. The doses are delivered at four week intervals. After four months, the animals receive a single immunization at two separate sites with 2mg of nucleic acid with or without 300 ìg of mutant HIV-1 envelope glycoprotein. This series may be followed by one or more subsequent recombinant protein subunit booster immunizations. The animals are bled at intervals of two to four weeks. Serum samples are prepared from each bleed to assay for the development of specific antibodies as described in the subsequent sections.

SHIV Challenge Experiments

Several chimeric HIV-SIV viruses have been created and characterized for infectivity in Rhesus monkeys. For Virus challenge experiments, the Rhesus monkeys are injected intravenously with a pre-titered dose of virus sufficient to infect greater than 9/10 animals. SHIV infection is determined by two assays. ELISA detection of SIV p27 antigen in monkey sera is determined using a commercially available kit (Coulter). Similarly, Western blot detection of anti-gag antibodies is performed using a commercially available kit (Cambridge Biotech). A reduction in either the rate of infection or the amount of p27

antigen produced in immunized versus control monkeys would indicate that the vaccine or vaccine combination has prophylactic value.

Part II

Synopsis of Results

The gp120 and gp41 subunits of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein associate via weak, non-covalent interactions, which can be stabilized by an intersubunit disulfide bond between cysteine residues introduced at appropriate sites in gp120 and gp41. The properties of such a protein, designated SOS gp140, are described herein. HIV-1$_{JR-FL}$ SOS gp140, proteolytically uncleaved gp140 (gp140$_{UNC}$) and gp120 were expressed in stably transfected Chinese hamster ovary (CHO) cells and analyzed for antigenic and structural properties before and after purification. In surface plasmon resonance (SPR) and radioimmunoprecipitation assays, SOS gp140 avidly bound the broadly neutralizing monoclonal antibodies (MAbs) 2G12 (anti-gp120) and 2F5 (anti-gp41), whereas gp140$_{UNC}$ bound these MAbs less avidly. In addition, MAb 17b against a CD4-induced epitope that overlaps the CCR5-binding site bound more strongly and rapidly to SOS gp140 than to gp140$_{UNC}$. In contrast, gp140$_{UNC}$ displayed the greater reactivity with non-neutralizing anti-gp120 and anti-gp41 MAbs. A series of immunoelectron microscopy studies suggested a model for SOS gp140 wherein the gp41 ectodomain (gp41$_{ECTO}$) occludes the "non-neutralizing" face of gp120, consistent with the antigenic properties of this protein. Also discussed is the application of Blue Native polyacrylamide gel electrophoresis (BN-PAGE), a high-resolution molecular sizing method, to the study of viral envelope proteins in purified and unpurified form. BN-PAGE and other biophysical studies demonstrated that SOS gp140 was monomeric, whereas gp140$_{UNC}$ comprised a mixture of non-covalently associated and disulfide-linked oligomers that could be resolved into dimers, trimers and tetramers by BN-PAGE. The oligomeric and antigenic properties of these proteins were largely unaffected by purification. An uncleaved gp140 protein containing the SOS cysteine mutations (SOS gp140$_{UNC}$) was also

oligomeric, indicating that cleavage of an oligomeric gp140 protein into gp120 and gp41 subunits destabilizes the gp41-gp41 interactions. This may be necessary for fusion to occur, but hinders the production of recombinant envelope glycoprotein complexes that mimic the native, virion-associated structure. Surprisingly, variable-loop-deleted SOS gp140 proteins were expressed as cleaved, non-covalently associated oligomers that were significantly more stable than the full-length protein. This suggests one path for producing proteolytically mature forms of the HIV-1 envelope glycoproteins in purified, oligomeric form. Overall, these findings have relevance for rational vaccine design.

Introduction

The native, fusion-competent form of the HIV-1 envelope glycoprotein complex is a trimeric structure composed of three gp120 and three gp41 subunits. The receptor-binding (CD4 and co-receptor) sites are located in the gp120 moieties, whereas the fusion peptides are located in the gp41 components (Chan, D.C., et al, Cell 89:263-273 (1997); Kwong, P.D., et al., Nature 393:648-659 (1998); Kwong, P.D., et al., J.Virol. 74:1961-1972 (2000); Poignard, P., et al., Annu.Rev.Immunol. 19:253-274 (2001); Tan, K., et al., Proc.Natl.Acad.Sci.U.S.A. 94:12303-12308 (1997); Weissenhorn, W., et al., Nature 387:426-430 (1997); Wyatt, R., et al., Nature 393:705-711 (1998)). In the generally accepted model of HIV-1 fusion, the sequential binding of gp120 to CD4 and a co-receptor induces a series of conformational changes in the gp41 subunits, leading to the insertion of the fusion peptides into the host cell membrane in a highly dynamic process (Doms, R.W. et al., J.Cell Biol. 151:F9-14 (2000); Jones, P.L., et al., J.Biol.Chem. 273:404-409 (1998); 34. Melikyan, G.B., et al., J.Cell Biol. 151:413-423 (2000); Sattentau, Q.J. et al., J.Exp.Med. 174:407-415 (1991); Sullivan, N., et al., J.Virol. 72:4694-4703 (1998); Trkola, A., et al., Nature 384:184-187 (1996); Wu, L., et al., Nature 384:179-183 (1996); Wyatt, R. et al., Science 280:1884-1888 (1998); Zhang, W., et al., Biochemistry 38:9405-9416 (1999)). The associations between the six components of the fusion-competent complex are maintained via non-covalent interactions between gp120 and gp41, and between the gp41 subunits (Poignard, P., et al., Annu.Rev.Immunol. 19:253-274 (2001); Wyatt, R. et al., Science 280:1884-1888 (1998)). These interactions are relatively weak, thus rendering the fusion-competent complex unstable. This instability perhaps facilitates the conformational changes in the various components that are

necessary for the fusion reaction to proceed efficiently, but it greatly complicates the task of isolating the native complex in purified form. The native complex thus falls apart before it can be purified, leaving only the dissociated subunits.

It is desirable to produce the native HIV-1 envelope complex to explore its potential as an immunogen, perhaps after modification to improve its exposure of critical neutralization epitopes. The limited neutralizing antibody response to HIV-1 in infected people is directed at the native complex, and is probably raised against it (Burton, et al., AIDS 11 Suppl A:S87-S98 (1997); Moore, J.P. et al., AIDS 9 Suppl A:S117-36 (1995); Parren, P.W.H.I. et al., Nat.Med. 3:366 (1997); Parren, P.W.H.I. et al., Adv.Immunol. 77:195-262 (2001); Parren, P.W.H.I., et al., J.Virol. 70:9046-9050 (1996); Parren, P.W.H.I., et al., AIDS 13 Suppl A:S137-S162 (1999)). In contrast, the isolated subunits have not proven efficient at inducing relevant neutralizing antibodies [reviewed in (Burton, et al., AIDS 11 Suppl A:S87-S98 (1997); Parren, P.W.H.I. et al., Adv.Immunol. 77:195-262 (2001); Parren, P.W.H.I., et al., AIDS 13 Suppl A:S137-S162 (1999))]. Therefore attempts have been made to make more stable forms of the envelope glycoprotein complex that better mimic the native structure. Usually, these efforts have focused on making various forms of soluble gp140 glycoproteins which contain gp120 but only the ectodomain of gp41 (Binley, J.M., et al., J.Virol. 74:627-643 (2000); Chen, B., et al. J.Biol.Chem. 275:34946-34953 (2000); Cherpelis, S.,et al., J.Virol. 75:1547-1550 (2001); Earl, P.L., et al., J.Virol. 68:3015-3026 (1994); Earl, P.L., et al., J.Virol. 75:645-653 (2001); Edinger, A.L., et al., J.Virol. 74:7922-7935 (2000); Farzan, M., et al., J.Virol. 72:7620-7625

(1998); Sanders, R.W., et al., J.Virol. 74:5091-5100 (2000); Stamatatos, L., et al., AIDS Res.Hum.Retroviruses 16:981-994 (2000); VanCott, T.C., et al., J.Virol. 71:4319-4330 (1997); Yang, X., et al., J.Virol. 74:5716-5725 (2000); Yang, X., et al., J.Virol. 74:4746-4754 (2000); Yang, X., et al., J.Virol. 75:1165-1171 (2001);Zhang, C.W., et al., J.Biol.Chem. 276:39577-39585 (2001)).

One approach to resolving the instability of the native complex is to remove the cleavage site that naturally exists between the gp120 and gp41 subunits. Doing so means that proteolysis of this site does not occur, leading to the expression of gp140 glycoproteins in which the gp120 subunit is covalently linked to the gp41 ectodomain ($gp41_{ECTO}$) by means of a peptide bond (Berman, P.W., et al., Nature 345:622-625 (1990); Berman, P.W.,et al., J.Virol. 62:3135-3142 (1988); Earl, P.L., et al., J.Virol. 71:2674-2684 (1997); Earl, P.L., et al., J.Virol. 68:3015-3026 (1994); Earl, P.L., et al., Proc.Nat.Acad.Sci.USA 87:648-652 (1990)). Such proteins can be oligomeric, and sometimes trimeric (Chen, B., et al. J.Biol.Chem. 275:34946-34953 (2000); Earl, P.L., et al., J.Virol. 71:2674-2684 (1997); Earl, P.L., et al., J.Virol. 68:3015-3026 (1994); Earl, P.L., et al., Proc.Nat.Acad.Sci.USA 87:648-652 (1990); Earl, P.L., et al., J.Virol. 75:645-653 (2001); Edinger, A.L., et al., J.Virol. 74:7922-7935 (2000); Farzan, M., et al., J.Virol. 72:7620-7625 (1998); Richardson, T.M.J., et al., J.Virol. 70:753-762 (1996); Stamatatos, L., et al., AIDS Res.Hum.Retroviruses 16:981-994 (2000); Yang, X., et al., J.Virol. 74:5716-5725 (2000); Yang, X., et al., J.Virol. 74:4746-4754 (2000); Yang, X., et al., J.Virol. 75:1165-1171 (2001);Zhang, C.W., et al., J.Biol.Chem. 276:39577-39585 (2001)). However, it is not clear that they truly represent

the structure of the native, fusion-competent complex in which the gp120-gp41 cleavage site is fully utilized. Hence the receptor-binding properties of uncleaved gp140 ($gp140_{UNC}$) proteins tend to be impaired, and non-neutralizing antibody epitopes are exposed on them that probably are not accessible on the native structure (Binley, J.M., et al., J.Virol. 74:627-643 (2000); Burton, et al., AIDS 11 Suppl A:S87-S98 (1997); Hoffman, T.L., et al., Proc.Natl.Acad.Sci.U.S.A. 97:11215-11220 (2000); Sattentau, Q.J., et al., Virology 206:713-717 (1995); Zhang, C.W., et al., J.Biol.Chem. 276:39577-39585 (2001)).

The present invention takes an alternative approach to the problem of gp120-gp41 instability, which is to retain the cleavage site but to introduce a disulfide bond between the gp120 and $gp41_{ECTO}$ subunits (Binley, J.M., et al., J.Virol. 74:627-643 (2000); Sanders, R.W., et al., J.Virol. 74:5091-5100 (2000)). Properly positioned, this intermolecular disulfide bond forms efficiently during envelope glycoprotein (Env) synthesis, allowing the secretion of gp140 proteins that are proteolytically processed but in which the association between the gp120 and $gp41_{ECTO}$ subunits is maintained by the disulfide bond.

It is shown herein that the gp41-gp41 interactions are unstable in the SOS gp140 protein, which is expressed and purified primarily as a monomer. In contrast, $gp140_{UNC}$ proteins B with or without the SOS cysteine substitutions B are multimeric, implying that cleavage of the peptide bond between gp120 and gp41 destabilizes the native complex. Despite being monomeric, the purified and unpurified forms of SOS gp140 are better antigenic structural mimics of the native, fusion-competent Env structure

than are the corresponding gp120 or $gp140_{UNC}$ proteins. This may be because the presence and orientation of $gp41_{ECTO}$ occludes certain non-neutralization epitopes on SOS gp140 while preserving the presentation of important neutralization sites. This explanation is consistent with immunoelectron microscopy studies of the protein. Unexpectedly, proteolytically mature, but variable-loop-deleted, SOS gp140 glycoproteins have enhanced oligomeric stability, such that these molecules warrant further study for their structural and immunogenic properties.

Materials and Methods

Plasmids

The pPPI4 eukaryotic expression vectors encoding SOS and uncleaved forms of HIV-1_{JR-FL} gp140 have been described previously (Binley, J.M., et al., J.Virol. 74:627-643 (2000); Trkola, A., et al., Nature 384:184-187 (1996)). The SOS gp140 protein contains cysteine substitutions at residues A501 in the C5 region of gp120 and T605 in gp41 (Binley, J.M., et al., J.Virol. 74:627-643 (2000); Sanders, R.W., et al., J.Virol. 74:5091-5100 (2000)). In $gp140_{UNC}$, the sequence KRRVVQREKRAV at the junction between gp120 and $gp41_{ECTO}$ has been replaced with a hexameric LR motif to prevent scission of gp140 into gp120 and $gp41_{ECTO}$ (Binley, J.M., et al., J.Virol. 74:627-643 (2000)). Plasmids encoding variable-loop-deleted forms of HIV-1_{JR-FL} SOS gp140 have been described (Sanders, R.W., et al., J.Virol. 74:5091-5100 (2000)). In these constructs, the tripeptide GAG is used to replace V1 loop sequences (D133-K155) and V2 loop sequences (F159-I194), alone or in combination. The SOS $gp140_{UNC}$ protein contains the same cysteine substitutions that are present in SOS gp140, but the residues REKR at the gp120-$gp41_{ECTO}$ cleavage site have been replaced by the sequence IEGR, to prevent gp140 cleavage. The furin gene (Thomas, G., et al., Science 241:226-230 (1988)) was expressed from plasmid pcDNA3.1furin (Binley, J.M., et al., J.Virol. 74:627-643 (2000)).

MAbs and CD4-based proteins

The following anti-gp120 MAbs were used: IgG1b12 [against the CD4 binding site (Burton, D.R., et al., Science 266:1024-1027 (1994))], 2G12 [against a unique C3-V4 glycan-dependent epitope (Trkola, A., et al., J.Virol. 70:1100-1108 (1996))], 17b [against

a CD4-inducible epitope (Thali, M. et al., J. Virol. 67:3978-3988 (1993)), 19b [against the V3 loop (Moore, J.P., et al. J. Virol. 69:122-130 (1995))], and 23A [against the C5 region (Moore, J.P., et al., J. Virol. 70:1863-1872 (1996))]. The anti-gp41 MAbs were 2F5 [against a cluster 1 epitope centered on the sequence ELDKWA (Muster, T., et al., J.Virol. 67:6642-6647 (1993), Parker, C.E., et al., J.Virol. 75:10906-10911 (2001))] and 2.2B [against epitope cluster II]. MAbs IgG1b12, 2G12 and 2F5 are broadly neutralizing (Trkola, A., et al., J.Virol. 69:6609-6617 (1995)). MAb 17b weakly neutralizes diverse strains of HIV-1, more so in the presence of soluble CD4 (Thali, M., et al., J.Virol. 67:3978-3988 (1993)), whereas the neutralizing activity of MAb 19b against primary isolates is limited (Trkola, A., et al., J.Virol. 72:1876-1885 (1998)). MAbs 23A and 2.2B are non-neutralizing. Soluble CD4 (sCD4) and the CD4-based molecule CD4-IgG2 have been described elsewhere (Allaway, G.P., et al., AIDS Res.Hum.Retroviruses 11:533-539 (1995)).

HIV-1 gp140 and gp120 glycoproteins

To create stable cell lines that secrete full-length HIV-1_{JR-FL} SOS gp140 or ΔV1V2 SOS gp140, DXB-11 dihydrofolate reductase (dhfr)-negative CHO cells were co-transfected with pcDNA3.1furin and either pPPI4-SOS gp140 (Binley, J.M., et al., J.Virol. 74:627-643 (2000)) or pPPI4-ΔV1V2* SOS gp140 (Sanders, R.W., et al., J.Virol. 74:5091-5100 (2000)), respectively, using the calcium phosphate precipitation method. Doubly transformed cells were selected by passaging the cells in nucleoside-free á-MEM media containing 10% fetal bovine serum (FBS), geneticin (Life Technologies, Rockville, MD) and methotrexate (Sigma, St. Louis, MO). The cells were amplified for gp140 expression by stepwise increases in methotrexate concentration, as described elsewhere

(Allaway, G.P., et al., AIDS Res.Hum.Retroviruses 11:533-539 (1995)). Clones were selected for SOS gp140 expression, assembly, and endoproteolytic processing based on SDS-PAGE and Western blot analyses of culture supernatants. CHO cells expressing SOS $gp140_{UNC}$ were created using similar methods, except that pcDNA3.1furin and geneticin were not used. Full-length SOS gp140 was purified from CHO cell culture supernatants by *Galanthus nivalis* lectin affinity chromatography (Sigma) and Superdex 200 gel filtration chromatography (Amersham-Pharmacia, Piscataway, NJ), as described elsewhere (Trkola, A., et al., Nature 384:184-187 (1996)). The $gp140_{UNC}$ glycoprotein was purified by lectin chromatography only. The concentration of purified Envs was measured by UV spectroscopy as described (Scandella, C.J., et al., AIDS Res. Hum. Retroviruses 9:1233-1244 (1993)), and was corroborated by ELISA and densitometric analysis of SDS-PAGE gels. Recombinant $HIV\text{-}1_{JR\text{-}FL}$, $HIV\text{-}1_{LAI}$ and $HIV\text{-}1_{YU2}$ gp120 glycoproteins were produced using methods that have been previously described (Trkola, A. et al., Nature 384:184-187 (1996); Wu, L., et al., Nature 384:179-183 (1996)).

Where indicated, HIV-1 envelope glycoproteins were transiently expressed in adherent 293T cells by transfection with Env- and furin-expressing plasmids, as described previously (Binley, J.M., et al., J.Virol. 74:627-643 (2000)). For radioimmunoprecipitation assays, the proteins were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine for 24 hours prior to analysis.

SDS-PAGE, radioimmunoprecipitation, Blue Native PAGE, and Western blot analyses

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed as described elsewhere (Binley,

J.M., et al., J.Virol. 74:627-643 (2000)). Reduced and non-reduced samples were prepared by boiling for 2 min in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) in the presence or absence, respectively, of 50 mM dithiothreitol (DTT). Protein purity was determined by densitometric analysis of the stained gels followed by the use of ImageQuant software (Molecular Devices, Sunnyvale, CA). Radioimmunoprecipitation assays (RIPA) were performed on Env-containing cell culture supernatants, as previously described (Binley, J.M., et al., J.Virol. 74:627-643 (2000); Sanders, R.W., et al., J.Virol. 74:5091-5100 (2000)).

Blue Native (BN)-PAGE was carried out with minor modifications to the published method (Schägger, H., et al., Analytical Biochemistry 217:220-230 (1994); Schägger, H. et al, Analytical Biochemistry 199:223-231 (1991)). Thus, purified protein samples or cell culture supernatants were diluted with an equal volume of a buffer containing 100mM 4-(N-morpholino)propane sulfonic acid (MOPS), 100 mM Tris-HCl, pH 7.7, 40% glycerol, 0.1% coomassie blue, just prior to loading onto a 4-12% Bis-Tris NuPAGE gel (Invitrogen). Typically, gel electrophoresis was performed for 2h at 150V (~0.07A) using 50mM MOPS, 50mM Tris, pH 7.7, 0.002% coomassie blue as cathode buffer, and 50 mM MOPS, 50 mM Tris, pH 7.7 as anode buffer. When purified proteins were analyzed, the gel was destained with several changes of 50mM MOPS, 50mM Tris, pH 7.7 subsequent to the electrophoresis step. Typically, 5 ìg of purified protein were loaded per lane.

For Western blot analyses, gels and polyvinylidine difluoride (PVDF) membranes were soaked for 10 minutes in transfer buffer

(192mM glycine, 25mM Tris, 0.05% SDS, pH 8.8 containing 20% methanol). Following transfer, PVDF membranes were destained of coomassie blue dye using 25% methanol and 10% acetic acid and air-dried. Destained membranes were probed using the anti-V3 loop MAb PA1 (Progenics) followed by horseradish peroxidase (HRP)-labeled anti-mouse IgG (Kirkegaard & Perry), each used at 0.2μg/mL final concentration. Luminometric detection of the envelope glycoproteins was obtained with the Renaissance Western Blot Chemiluminescence Reagent *Plus* system (Perkin Elmer Life Sciences, Boston, MA). Bovine serum albumin (BSA), apo-ferritin, and thyroglobulin were obtained from Amersham Biosciences (Piscataway, NJ) and used as molecular weight standards.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

Proteins were dialyzed overnight against water prior to analysis. Where indicated, SOS gp140 (1mg/ml) was reduced with 10mM DTT (Sigma), after which iodoacetamide (Sigma) was added to a final concentration of 100mM, before dialysis. The samples were mixed with an equal volume of sinapinic acid matrix solution, dried at room temperature, and analyzed by MALDI-TOF mass spectrometry (Lewis, J.K., et al., Proc.Nat.Acad.Sci.USA 95:8596-8601 (1998)). MALDI-TOF mass spectra were acquired on a PerSeptive Biosystems Voyager-STR mass spectrometer with delayed extraction. Samples were irradiated with a nitrogen laser (Laser Science Inc.) operated at 337nm. Ions produced in the sample target were accelerated with a deflection voltage of 30,000V.

Sedimentation equilibrium analysis

Sedimentation equilibrium measurements were performed on a Beckman XL-A Optima analytical ultracentrifuge with an An-60 Ti

rotor at 20EC. Protein samples were dialyzed overnight against 50mM sodium phosphate (pH 7.0) and 150mM NaCl, loaded at initial concentrations of 0.25mM, 0.5mM and 1mM, then centrifuged in a six-sector cell at rotor speeds of 6,000 and 9,000 rpm. Data were acquired at two wavelengths per rotor speed and processed simultaneously with a nonlinear least squares fitting routine (Johnson, M.L. et al., Biophys.J. 36:575-588 (1981)). Solvent density and protein partial specific volume were calculated according to solvent and protein composition, respectively (Laue, T.M., et al. In S.E. Harding, et al. Royal Society of Chemistry, Cambridge (1992)).

Size exclusion chromatography

Purified, CHO cell-expressed SOS gp140, $gp140_{UNC}$ and gp120 proteins were analyzed by size exclusion chromatography on a TSK $G3000SW_{XL}$ HPLC column (TosoHaas, Montgomeryville, PA) using phosphate buffered saline (PBS) as the running buffer. The protein retention time was determined by monitoring the UV absorbance of the column effluent at a wavelength of 280 nm. The column was calibrated using ferritin as a model protein that exists in oligomeric states of 220 kDa, 440 kDa and 880 kDa (Gerl, M., et al., Biochemistry 27:4089-4096 (1988)).

Surface plasmon resonance measurements

A Biacore X optical biosensor was used. Each Mab was immobilized at 8000-10,000 resonance units by the amine coupling method to a CM5 sensor chip, according to the manufacturer=s instructions (Biacore, Inc., Piscataway, NJ). A reference surface (lacking antibody) was used as a background control. Binding experiments were performed at 25°C in HSB-EP buffer (10nM HEPES pH 7.4, 150mM

NaCl, 3mM EDTA, 0.005% (v/v) Surfactant P20). Purified Envs (25 nM) were run over the test and control chips at a flow rate of 30μl/min, whereas CHO cell culture supernatants (~5 nM Env) were analyzed at 10μl/min. To study the exposure of CD4-induced (CD4i) epitopes, sCD4 was added to the envelope glycoproteins at an 8-molar excess concentration for at least 1 hour prior to analysis. The sensor surface was regenerated with a short pulse of 3.5M $MgCl_2$.

Immunoelectron microscopy

Immunoelectron-microscopic analyses of SOS gp140 and gp120 alone and in complex with MAb, MAb fragments and sCD4 were performed by negative staining with uranyl formate as previously described (Roux, K.H., Methods Enzymol. 178:130-144 (1989; ,Roux, K.H., Methods 10:247-256 (1996)). The samples were examined on a JEOL JEM CX-100 electron microscope and photographed at 100,000 diameters magnification.

Immune complex image digitalizing and averaging

The electron micrographs of immune complex images were digitalized on an AGFA DUOSCAN T2500 Negative Scanner (Ridgefield Park, NJ). Potentially informative complexes were selected and windowed as 256 X 256 pixel images. These randomly oriented complexes were then brought into approximate alignment utilizing the multi-reference alignment function of the SPIDER program (Frank, J., et al., J.Struct.Biol. 116:190-199 (1996)). The aligned images were subsequently averaged to improve the signal-to-noise ratio.

Molecular modeling

The SwissPDBviewer program (Guex, N. et al., Electrophoresis

18:2714-2723 (1997)) was used to enhance the EM-based interpretations and to investigate the likely location of the gp41 domain in SOS gp140.

RESULTS

Assembly and cleavage of purified SOS gp140

The antigenic properties of unpurified HIV-1_{JR-FL} SOS gp140 proteins produced via transient transfection of 293T cells have been previously described (see Binley, J.M., et al., J.Virol. 74:627-643 (2000)). To facilitate preparation of larger amounts of this protein for evaluation in purified form, a stable CHO cell line was constructed that expresses both SOS gp140 and human furin. Heterologous furin was expressed to facilitate efficient proteolytic processing of SOS gp140 (Binley, J., et al., [published erratum appears in Nature 1997 Sept 11;389(6647):131], Nature 387:346-348 (1997)).

The SOS gp140 protein was purified from CHO cell supernatants to ~90% homogeneity (Fig. 16, Lane 8). Only minor amounts of free gp120 were present in the SOS gp140 preparation, indicating that the inter-subunit disulfide bond remained substantially intact during purification. No high molecular weight SOS gp140 oligomers or aggregates were observed (Fig. 16, Lane 8). Under non-reducing conditions, SOS gp140 migrated as a predominant 140 kDa band. The major contaminant was bovine alpha 2-macroglobulin, which migrates as an ~170 kDa band on a reducing SDS-PAGE gel (Fig. 16, Lane 3) and can be eliminated by adaptation of the CHO cell line to serum-free culture. Upon reduction with DTT, the purified SOS gp140 protein migrated as a predominant 120 kDa band, with a minor (~10%) fraction of the 140 kDa band present (Fig. 16, Lane 3). These data indicated that approximately 90% of the SOS gp140 protein was proteolytically processed.

The HIV-1_{JR-FL} $gp140_{UNC}$ protein was expressed in CHO cells using

similar methods, although without co-transfected furin, and was also obtained at ~90% purity. It too contained alpha 2-macroglobulin as the major contaminant, but no free gp120 was detectable (Fig. 16, Lanes 4 and 9). In the absence of DTT, alpha 2-macroglobulin migrates as a ~350 kDa dimer and is not clearly resolved from gp140$_{UNC}$ oligomers (Fig. 16, Lane 9). Under non-reducing conditions, bands consistent with gp140$_{UNC}$ monomers (140 kDa), dimers (280 kDa), and trimers (420 kDa) were observed in roughly equal amounts (Fig. 16, Lane 9). These proteins were reactive with anti-gp120 MAbs in Western blot analysis. When treated with DTT, gp140$_{UNC}$ gave rise to an intensified monomer band at 140 kDa and an alpha 2-macroglobulin monomer band at ~170 kDa; but gp140 oligomers were absent (Fig. 16, compare Lanes 4 and 9). Thus, disulfide-linked, reducible oligomers comprise half or more of the gp140$_{UNC}$ preparation. Comparable amounts of reducible oligomers have been observed in gp140$_{UNC}$ protein preparations derived from subtype A, B and E viruses, with minor strain-to-strain differences (Owens, R.J. et al., Virology 179:827-833 (1999); Staropoli, I., et al., J.Biol.Chem. 275:35137-35145 (2000)). Reducible gp160 oligomers of this type have been proposed to contain aberrant intermolecular disulfide bonds (Owens, R.J. et al., Virology 179:827-833 (1999)). If so, at least some of the oligomers present in gp140$_{UNC}$ preparations represent misfolded protein aggregates.

Biophysical properties of purified SOS gp140

Matrix-assisted laser desorption ionization mass spectrometry

This technique was used to determine the absolute molecular masses of HIV-1$_{JR-FL}$ gp120 and SOS gp140. As indicated in Table 1 (shown below), the measured molecular masses were 121.9 kDa for

SOS gp140 and 91.3 kDa for gp120.

HIV-1_{JR-FL} envelope glycoprotein	mass, kDa
gp120	91.3
SOS gp140	121.9
SOS gp140, reduced:	
uncleaved gp140	118.5
gp120	91.8
$gp41_{ECTO}$	27.0

Table 1. Molecular masses of recombinant HIV-1_{JR-FL} envelope glycoproteins as determined by MALDI-TOF mass spectrometry.

As shown in table 1, reduced SOS gp140 gave rise to a small peak of uncleaved gp140 at 118.5 kDa, a gp120 peak at 91.8 kDa and a $gp41_{ECTO}$ peak at 27 kDa. Differences in glycosylation between cleaved and uncleaved SOS gp140 proteins could account for the 3.4 kDa difference in their measured masses. A smaller difference (~500 Da) was observed in the mass of gp120 when it was expressed alone and in the context of SOS gp140. The alanine-cysteine SOS mutation would be expected to increase the mass of gp120 by only 32 Da (one sulfur atom), so again a minor difference in glycosylation patterns may be responsible. The measured mass of HIV-1_{JR-FL} gp120 is comparable to previously reported molecular masses of CHO cell-expressed HIV-1_{GB8} gp120 (91.8 kDa) and

Drosophila cell-expressed HIV-1_{WD61} gp120 (99.6 kDa) (Jones, D.H., et al., Vaccine 13:991-999 (1995)); Myszka, D.G., et al., Proc.Natl.Acad.Sci.U.S.A. 97:9026-9031 (2000)). The anomalously high molecular weights (~120 kDa and ~140 kDa, respectively, Fig. 16) observed for gp120 and SOS gp140 by SDS-PAGE reflect the high carbohydrate content of these proteins. The extended structure of the glycans and their poor reactivity with the dodecyl sulfate anion retard the electrophoretic migration of the glycoproteins through SDS-PAGE gel matrices (Jones, D.H., et al., Vaccine 13:991-999 (1995)).

Ultracentrifugation:

Sedimentation equilibrium measurements were used to examine the oligomeric state of purified SOS gp140. Over protein concentrations ranging from 0.25-1.0mM, the apparent molecular weight of SOS gp140 was consistently found to be 155 kDa (Fig. 17A). Hence, the purified SOS gp140 protein is monomeric in solution. There was no systematic dependence of molecular weight on protein concentration over the range studied. However, the residuals (the difference between the data and the theoretical curve for a monomer) deviated from zero in a systematic fashion (Fig. 17A), suggesting the presence of small amounts of oligomeric material.

Analytical gel filtration chromatography :

Purified HIV-1_{JR-FL} SOS gp140, $gp140_{UNC}$ and gp120 proteins were also examined using size exclusion chromatography. Monomeric gp120 eluted with a retention time of 6.24 min and an apparent molecular weight of ~200 kDa (Fig. 17B). The apparently large size of this protein reflects the extended structures of its

carbohydrate moieties. The retention time (5.95 min) and apparent molecular weight (~220 kDa) of the SOS gp140 protein are consistent with it being a monomer that is slightly larger than gp120. In contrast, the $gp140_{UNC}$ protein eluted at 4.91 min as a broad peak with an average molecular weight of >500 kDa, which is consistent with it comprising a mixture of oligomeric species. Although the chromatogram suggests the existence of multiple species in the $gp140_{UNC}$ preparation, this gel-filtration technique cannot resolve mixtures of gp140 dimers, trimers and tetramers.

Blue Native polyacrylamide gel electrophoresis :

BN-PAGE was used to examine the oligomeric state of the purified SOS gp140 and $gp140_{UNC}$ proteins. In BN-PAGE, most proteins are fractionated according to their Stokes' radius (Schagger, H., et al., Analytic Biochemistry 217:220-230 (1994); Schagger, H. et al., Analytic biochemistry 199:223-231 (1991)). This technique was applied first to a model set of soluble proteins, including gp120 alone and in complex with sCD4 (Fig. 17C). The model proteins included thyroglobulin and ferritin, which naturally comprise a distribution of non-covalent oligomers of varying size. The oligomeric states of these multi-subunit proteins, as determined by BN-PAGE, are similar to those observed using other non-denaturing techniques ((Gerl, M., et al., Biochemistry 27:4089-4096 (1988); Venkatesh, S.G. et al., Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol. 122:13-20 (1999)). BSA exists as monomers, dimers, and higher order species in solution (Lambin, P., et al., Rev.Fr.Transfus.Immunohematol. 25:487-498 (1982)); the same ladder of oligomers was observed in BN-PAGE. Not surprisingly, the gp120/sCD4 complex, which has an association constant in the nanomolar range (Allaway, G.P., et al., AIDS Res.Hum.Retroviruses 11:533-539 (1995)), remained

intact during BN-PAGE analysis.

The purified SOS gp140 protein was largely monomeric by BN-PAGE (Fig. 17D), although a minor amount (<10%) of dimeric species was also observed. The purified $gp140_{UNC}$ protein migrated as well-resolved dimers, trimers and tetramers, with trace amounts of monomer present (Fig. 17D). The $gp140_{UNC}$ dimer represented the major oligomeric form of the protein present under non-denaturing conditions. Although tetrameric $gp140_{UNC}$ is a distinct minor species on BN-PAGE gels (Fig. 17D), it is absent from non-reduced SDS-PAGE gels (Fig. 16). Upon treatment with SDS and heat, the $gp140_{UNC}$ tetramers probably revert to lower molecular weight species, such as monomers and/or disulfide-linked dimers. As expected, HIV-1_{JR-FL} gp120 migrated as a predominant 120 kDa monomeric protein. BN-PAGE analyses of unpurified gp140 proteins are described below (see Fig. 23).

Overall, ultracentrifugation, gel filtration and BN-PAGE analyses were in excellent agreement as to the oligomeric states of these purified Env proteins. BN-PAGE, however, was the only method capable of clearly resolving the mixture of oligomeric species contained in the $gp140_{UNC}$ preparation.

Immunoelectron microscopy of SOS gp140

SOS gp140 and SOS gp140-MAb complexes:

In the absence of antibodies, the electron micrographs revealed SOS gp140 to be mostly monomeric, randomly oriented and multi-lobed (Fig 18A). Qualitatively similar images were obtained with HIV-1_{JR-FL} gp120, and the two proteins could not be clearly

distinguished in the absence of MAbs or other means of orienting the images.

Electron micrographs were also obtained of SOS gp140 in complex with MAbs 2F5 (Fig. 18B), IgG1b12 (Fig. 18C) and 2G12 (Fig. 18D). To aid in interpretation, the complexes were masked and rotated such that the presumptive Fc of the MAb points downward. Schematic diagrams are also provided for each complex in order to illustrate the basic geometry and stoichiometry observed. In each case, the complexes shown represent the majority or plurality species present. However, other species, such as free MAb and monovalent MAb-SOS gp140 complexes, were also present in each sample .

When combined with IgG1b12 or 2F5, SOS gp140 formed rather typical immune complexes composed of a single MAb and up to two SOS gp140s (Fig. 18B and 18C). The complexes adopted the characteristic Y-shaped antibody structure, with a variable angle between the Fab arms of the MAb. In contrast, the 2G12/SOS gp140 complexes produced strikingly different images (Fig. 18D). Y-shaped complexes comprising two distinct Fab arms with bound SOS gp140s were rare. Instead the 2G12-SOS gp140 images were strongly linear and appeared to represent one MAb bound to two SOS gp140 proteins aligned in parallel. The parallel alignment of the SOS gp140s forces the two Fab arms into similar alignment, resulting in an overall linear structure. These complexes are unprecedented in the immunoelectron microscopy studies of Env-MAb complexes (Roux, K.H., Methods Enzymol. 178:130-144 (1989); Roux, K.H., Methods 10:247-256 (1996); Zhu, P., et al., J.Virol. 75:6682-6686 (2001)). Of note is that the HIV-1_{JR-FL} gp120-2G12 complexes do not adopt this parallel configuration but instead resemble the SOS

gp140-2F5 and SOS gp140-IgG1b12 complexes. One hypothesis is that 2G12 binds to SOS gp140 in an orientation that promotes residual weak interactions between the $gp41_{ECTO}$ moieties, which then stabilize the complex in the parallel configuration observed.

Combinations of the above, well-characterized MAbs were used to examine the relative placement of their epitopes on SOS gp140. In the first combination, SOS gp140-2F5-IgG1b12, multiple ring structures were observed which appeared to be composed of two SOS gp140 proteins bridged by two antibody molecules. To distinguish between the 2F5 and IgG1b12 MAbs, complexes formed between IgG1b12 $F(ab')_2$, SOS gp140 and the intact 2F5 Mab were examined. Characteristic ring structures were again observed (Fig. 18E). The ring complexes were then subjected to computational analysis using the SPIDER program package to yield several categories of averaged images. The MAb 2F5 and IgG1b12 $F(ab')_2$ components can clearly be delineated in the images, as can the SOS gp140 molecule. When bound to a given SOS gp140 molecule, the Fab arms of 2F5 and IgG1b12 lie at approximately right angles, as indicated in the schematic diagram (Fig. 18E).

In marked contrast to the IgG1b12-containing ternary complexes, those composed of SOS, 2F5 and 2G12 formed extended chains rather than closed rings (Fig. 18F). These observations place the 2F5 and 2G12 epitopes at opposite ends of the SOS gp140 molecule. There was significant heterogeneity in the stoichiometry of the 2F5/2G12/SOS gp140 complexes, just one example of which is indicated in the schematic diagram.

Immunoelectron microscopy of SOS gp140 and gp120 in complex with

sCD4 and MAb 17b:

In an effort to further characterize the topology of SOS gp140, it was reacted with MAb 17b and/or sCD4 (Fig. 19). The corresponding YU2 gp120 complexes were generated for comparison. As expected, the combination of MAb 17b plus SOS gp140 or gp120 alone did not form complexes, consistent with the need for sCD4 to induce the 17b epitope. Similarly, unremarkable complexes were obtained when sCD4 was mixed with SOS gp140 or gp120 in the absence of MAb 17b . However, complexes with clearly defined geometry were obtained for sCD4/Env/17b (Fig. 19A and 19B).

These complexes were composed of 17b with one or two attached SOS gp140s or gp120s, together with tangentially protruding sCD4 molecules. These complexes were then subjected to computer-assisted averaging (Fig. 19C and 19F). The free arm and the Fc region of MAb 17b were disordered in these images due to the flexibility of the MAb, so the averaged images were masked to highlight the better-resolved sCD4, Env and 17b Fab structures (Fig. 19D and 19G). The gp120 and SOS gp140 images were qualitatively similar, but an image subtraction of one from the other revealed the presence of additional mass on the SOS gp140 protein (arrowed in Fig. 19D and 19E). This additional mass may represent $gp41_{ECTO}$, although other explanations, such as differences in the primary sequence and/or glycosylation of the gp120 and SOS gp140 proteins used, cannot be strictly excluded.

In order to orient the putative $gp41_{ECTO}$ moiety in relation to the remaining structures seen in the electron micrographs, the X-ray structure of the gp120 core in complex with the D1D2 domain of sCD4 and Fab 17b (Kwong, P.D., et al., Nature 393:648-659 (1998)) was docked, using Program O, into the profile map

obtained for the sCD4/gp120/MAb 17b complex (Fig. 19H). Given that there are differences in the gp120 (whole *vs.* core) and CD4 (four domain *vs.* two domain) molecules used for the electron microscopy and crystallization studies, there is reasonable agreement in the overall topology of the structures generated.

This agreement in structures (Fig. 19H) enabled the positioning of the putative $gp41_{ECTO}$ moiety in relation to the core gp120 structure (Fig. 20). The previously defined neutralizing, non-neutralizing, and silent faces of gp120 (Moore, J.P. et al., J.Virol. 70 :1863-1872 (1996); Wyatt, R., et al., Nature 393:705-711 (1998)) are illustrated, as are the IgG1b12 ((Saphire, E.O., et al., Science 293:1155-1159 (2001)) and 2G12 (Wyatt, R., et al., Nature 393:705-711 (1998)) epitopes. According to this model, the $gp41_{ECTO}$ moiety recognized by MAb 2F5 is located at ~90B relative to the IgG1b12 epitope and ~180B from the 2G12 epitope (Fig. 20B). This model is in broad agreement with the independently derived electron microscopy images of the complexes formed between SOS gp140 and combinations of these MAbs (Fig. 18E and 18F). This putative placement of $gp41_{ECTO}$ would cause it to largely occlude the non-neutralizing face of gp120, a result that is consistent with the MAb reactivity patterns observed for SOS gp140 both in the present invention and elsewhere (Binley, J.M., et al., J.Virol. 74:627-643 (2000)).

<u>Antigenic properties of unpurified SOS gp140 and $gp140_{UNC}$ proteins</u>

<u>Radioimmunoprecipitation assays:</u>

RIPA was used to determine whether the antigenicity of $HIV\text{-}1_{JR\text{-}FL}$ SOS gp140 differed when the protein was expressed in stably

transfected CHO cells, compared to what was observed previously when the same protein was expressed in transiently transfected 293T cells (Binley, J.M., et al., J.Virol. 74:627-643 (2000)). The SOS gp140 proteins in unpurified supernatants expressed from CHO cells were efficiently recognized by neutralizing agents to gp120 epitopes located in the C3/V4 region (MAb 2G12), the CD4 binding site (the CD4-IgG2 molecule), and the V3 loop (MAb 19b) (Fig. 21). In addition, the conserved CD4-induced neutralization epitope defined by MAb 17b was strongly induced on SOS gp140 by sCD4. SOS gp140 was also efficiently immunoprecipitated by the broadly neutralizing gp41 MAb 2F5. In contrast, SOS gp140 was largely unreactive with the non-neutralizing MAbs 23A and 2.2B to gp120 and gp41, respectively (Fig. 21, Lanes 3 and 9). A comparison of these analyses with previous observations (Binley, J.M., et al., J.Virol. 74:627-643 (2000)) indicates that CHO and 293T cell-derived HIV-1_{JR-FL} SOS gp140 proteins possess similar antigenic properties.

Relatively minor amounts of free gp120 were observed in the unpurified SOS gp140 CHO cell supernatants (Fig. 21, Lanes 1, 5, 7, 8). This free gp120 was preferentially recognized by MAb 23A, suggesting that its C5 epitope is largely obscured in SOS gp140 (Fig. 21, Lane 9). This is consistent with the electron microscopy-derived topology model described above (Fig. 20B), and with what is known about the gp120-gp41 interface (Helseth, E., et al., J.Virol. 65:2119-2123 (1991); Moore, J.P. et al., J.Virol. 70 :1863-1872 (1996); Wyatt, R., et al., J.Virol. 71:9722-9731 (1997)). Processing of SOS gp140 at the gp120-gp41 cleavage site was efficient, as determined by RIPAs performed under reducing and non-reducing conditions (Fig. 21, compare Lanes 1 and 2). Similar levels of assembly and proteolytic

processing were observed when unpurified SOS gp140 was analyzed by Western blotting rather than RIPA. These findings also are comparable to those seen with 293T cell-derived HIV-1_{JR-FL} SOS gp140 (Binley, J.M., et al., J.Virol. 74:627-643 (2000)). Thus the folding, assembly, and processing of this protein appear to be largely independent of the cell line used for its production.

Surface plasmon resonance assays:

SPR was used to further characterize the antibody and receptor-binding properties of unpurified, CHO cell-expressed SOS gp140 and $gp140_{UNC}$ proteins. A comparison of results obtained using SPR and RIPA with the same MAbs allows one to determine if the antigenicity of these proteins is method-dependent. Whereas SPR is a kinetically-limited procedure that is completed in one or more minutes, RIPA is an equilibrium method in which Env-MAb binding occurs over several hours. SPR analysis was also performed on purified and unpurified forms of the SOS gp140 and $gp140_{UNC}$ proteins, to assess whether protein antigenicity was significantly altered during purification. Purified HIV-1_{JR-FL} gp120 was also studied. Although the purified SOS gp140 protein is a monomer, it does contain the gp120 subunit linked to the ectodomain of gp41. Since there is evidence that the presence of gp41 can affect the antigenic structure of gp120 (Klasse, P.J., et al., Virology 196:332-337 (1993); Reitz, M.S.J., et al., Cell 54:57-63 (1988),72), it was thought to be worth determining whether monomeric SOS gp140 behaved differently than monomeric gp120 in its interactions with neutralizing and non-neutralizing Mabs.

There was good concordance of results between RIPA- (Fig. 21) and

SPR-based (Fig. 22) antigenicity analyses of unpurified SOS gp140 in CHO cell supernatants. For example, SOS gp140 bound the broadly neutralizing anti-gp41 MAb 2F5 (Fig. 21, Lane 4 and Fig. 22B) but not the non-neutralizing anti-gp41 MAb 2.2B (Fig. 21, Lane 3 and Fig. 22D). Similarly, binding of MAb 17b was strongly potentiated by sCD4 (Fig. 21, Lanes 6-7 and Fig. 22F). Unpurified SOS gp140 bound the neutralizing anti-gp120 MAbs 2G12 and 19b, but not the non-neutralizing anti-gp120 MAb 23A in both SPR and RIPA (Fig. 21, Lanes 1, 8, 9) experiments. Taken together, the RIPA and SPR data indicate that unpurified, CHO cell-derived SOS gp140 rapidly and avidly binds neutralizing anti-gp120 and anti-gp41 MAbs, whereas binding to the present set of non-neutralizing MAbs is not measurable by either technique.

SPR revealed some significant differences in the reactivities of SOS gp140 and $gp140_{UNC}$ proteins with anti-gp41 MAbs. Thus, SOS gp140, but not $gp140_{UNC}$, bound MAb 2F5 but not MAb 2.2B, whereas the converse was true for $gp140_{UNC}$. Notable, albeit less dramatic, differences were observed in the reactivity of SOS gp140 and $gp140_{UNC}$ with some anti-gp120 MAbs. Of the two proteins, SOS gp140 had the greater kinetics and magnitude of binding to the neutralizing MAbs IgG1b12 (Fig. 22G), 2G12 (Fig. 22H) and 17b in the presence of sCD4 (Fig. 22E, 22F). The binding of $gp140_{UNC}$ to 17b was clearly potentiated by sCD4, as has been reported elsewhere (Zhang, C.W., et al., J.Biol.Chem. 276:39577-39585 (2001)). Neither SOS gp140 nor $gp140_{UNC}$ bound the anti-gp120 MAb 23A. This was expected for $gp140_{UNC}$ since the C5 amino acid substitutions that eliminate the cleavage site directly affect the epitope for MAb 23A (Moore, J.P., et al., J.Virol. 68:6836-6847(1994)).

Qualitatively, the antigenicities of SOS gp140 and $gp140_{UNC}$ were little changed upon purification (Fig. 22, compare panels A, C and E with panels B, D and F). Hence the lectin affinity and gel filtration columns used for purification do not appear to significantly affect, or select for, a particular conformational state of these proteins. However, these studies do not allow for direct, quantitative comparisons of SPR data derived using purified and unpurified materials.

Compared with monomeric gp120, the purified $gp140_{UNC}$ protein reacted more strongly with MAb 2G12 but less strongly with MAb IgG1b12. Prior SPR studies have demonstrated that 2G12 avidly binds to oligomeric forms of Env (Zeder-Lutz, G. et al., European Journal of Biochemistry 268:2856-2866 (2001)), and it is possible that MAb 2G12 is capable of undergoing bivalent binding to oligomeric Envs.

Oligomeric properties of unpurified SOS gp140 and $gp140_{UNC}$ proteins

BN-PAGE was used to examine the oligomeric state of the SOS gp140 and $gp140_{UNC}$ proteins present in freshly prepared, CHO cell culture supernatants. The SOS gp140 protein was largely monomeric by BN-PAGE, with only a minor proportion of higher order proteins present (Fig. 23A). In some, but not all, 293T cell preparations, greater but highly variable amounts of dimers and higher-order oligomers were observed using BN-PAGE (see Fig.23B). This probably accounts for the previous report that oligomers can be observed in unpurified SOS gp140 preparations using other techniques (Binley, J.M., et al., J.Virol. 74:627-643 (2000)).

The unpurified $gp140_{UNC}$ protein typically migrated as well-resolved dimers, trimers and tetramers, with trace amounts of monomer sometimes present (Fig. 23A). Qualitatively similar banding patterns were observed for purified (Fig. 17D) and unpurified $gp140_{UNC}$ proteins (Fig. 23A). In each case, dimers of $gp140_{UNC}$ were the most abundant oligomeric species. $HIV\text{-}1_{CR\text{-}FL}$ gp120 ran as a predominant 120 kDa monomeric band, although small amounts of gp120 dimers were observed in some unpurified supernatants. In general, the BN-PAGE analyses indicate that the oligomeric properties of the various Env proteins did not change appreciably upon purification (compare Fig. 23A and Fig. 17D).

The same CHO cell supernatants were also analyzed by analytical gel filtration, the column fractions being collected in 0.2 mL increments and analyzed for Env content by Western blotting. The retention times of unpurified gp120, SOS gp140 and $gp140_{UNC}$ proteins were determined to be ~6.1, ~5.9 and ~5.2 min, respectively. These values agree with those observed for the purified proteins (Fig. 2B) to within the precision of the method. The gel filtration studies thus corroborate the BN-PAGE data in that unpurified gp120 and SOS gp140 were mostly monomeric, while $gp140_{UNC}$ was mostly oligomeric . However, unlike BN-PAGE, this analytical gel filtration procedure does not have sufficient resolving power to characterize the distribution of the oligomeric species present in the $gp140_{UNC}$ preparation.

SDS-PAGE followed by Western blot analyses of supernatants containing unpurified SOS gp140 and $gp140_{UNC}$ proteins yielded banding patterns similar to those shown in Fig. 16 for the purified proteins . The gp120 preparation contained ~10% dimer, which was observed only when SDS-PAGE analyses were carried out

under non-reducing conditions. Thus the gp120 dimer represents disulfide-linked and presumably misfolded material (Owens, R.J. et al., Virology 179:827-833 (1999)).

Variable loop-deleted SOS gp140 glycoproteins form more stable oligomers

HIV-1_{JR-FL} SOS gp140 glycoproteins from which one or more of the gp120 variable loops were deleted to better expose underlying, conserved regions around the CD4- and coreceptor-binding sites have been previously described. It was possible to remove the V1, V2 and V3 loop structures individually or in pairs without adversely affecting the formation of the intersubunit disulfide bond, proper proteolytic cleavage, or protein folding. However, the triple loop-deletant was not efficiently cleaved (Sanders, R.W., et al., J.Virol. 74:5091-5100 (2000)). In order to explore the oligomeric properties of these modified SOS gp140 glycoproteins, the supernatants of 293T cells transiently co-transfected with these gp140 constructs and furin were analyzed by BN-PAGE. Unexpectedly, deletion of the variable loops, both alone and in combination, significantly enhanced the stability of the SOS gp140 oligomers. The ΔV1V2 SOS gp140 preparation contained almost exclusively trimeric and tetrameric species, whereas ΔV1 SOS gp140 formed a mixture of dimers, trimers and tetramers similar to that seen with $gp140_{UNC}$. The ΔV2 SOS gp140 protein was predominantly oligomeric, but it also contained significant quantities of monomer. Thus, in terms of oligomeric stability, the SOS proteins can be ranked as follows: ΔV1V2 SOS gp140 > ΔV1 SOS gp140 > V2 SOS gp140 > full-length SOS gp140. The

reasons for this rank order are not yet clear, but are under investigation.

Based on the above observations, it was decided generate a CHO cell line that stably expresses the ΔV1V2 SOS gp140 protein. Supernatants from the optimized CHO cell line were first analyzed by SDS-PAGE under reducing and non-reducing conditions, followed by Western blot detection. The major Env band was seen at 120 kDa (ΔV1V2 gp140 protein) in the non-reduced gel and at 100 kDa (ΔV1V2 gp120 protein) in the reduced gel . These results are consistent with the prior findings that deletion of the V1V2 loops decreases the apparent molecular weight of the protein by ~20 kDa (Sanders, R.W., et al., J. Virol. 74:5091-5100 (2000)). Notably, the ΔV1V2 SOS gp140 protein was largely free both of disulfide-linked aggregates and of the ~100 kDa loop-deleted, free gp120 protein. Thus proteolytic cleavage and SOS disulfide bond formation occur efficiently in the ΔV1V2 SOS gp140 protein .

CHO cell supernatants containing ΔV1V2 SOS gp140, full-length SOS gp140 and $gp140_{UNC}$ were also analyzed by BN-PAGE and Western blotting (Fig. 23A). As was observed with the transiently transfected 293T cells, unpurified CHO cell-derived material was oligomeric. The CHO cell-derived ΔV1V2 SOS gp140 migrated as a distinct single band with a molecular weight consistent with that of a trimer (360 kDa); the ΔV1V2 SOS gp140 band lies between those of $gp140_{UNC}$ dimer (280 kDa) and $gp140_{UNC}$ trimer (420 kDa) (Fig. 8A). Hence the ΔV1V2 SOS gp140 protein represents a proteolytically mature form of HIV-1 Env that oligomerizes into presumptive trimers via non-covalent interactions.

The uncleaved SOS gp140 and $gp140_{UNC}$ proteins possess similar oligomeric properties

Overall, the above analyses reveal a clear difference in the oligomeric properties of the SOS gp140 and $gp140_{UNC}$ proteins. One structural difference between these proteins is their proteolytic cleavage status, another is the presence or absence of the intersubunit disulfide bond that defines SOS gp140 proteins. To address the question of whether it is gp120-gp41 cleavage or the introduced cysteine residues that destabilize the SOS gp140 oligomers, the SOS $gp140_{UNC}$ protein was produced. Here, the cysteines capable of intersubunit disulfide bond formation are present, but the cleavage site between gp120 and $gp41_{ECTO}$ has also been modified to prevent cleavage. The SOS $gp140_{UNC}$, SOS gp140 and $gp140_{UNC}$ proteins were all expressed transiently in 293T cells and analyzed by BN-PAGE (Fig. 23B). In this and multiple repeat experiments, SOS $gp140_{UNC}$ and $gp140_{UNC}$ had similar migration patterns on the native gel, with the dimer band predominating and some monomers, trimers and tetramers also present. In contrast, SOS gp140 was primarily monomeric, although small amounts of dimeric and trimeric species were also observed in this particular analysis (Fig. 23B).

The above results suggest that the SOS $gp140_{UNC}$ protein behaves more like the $gp140_{UNC}$ protein than the SOS gp140 protein. This, in turn, implies that the cleavage of gp140 into gp120 and $gp41_{ECTO}$ has a substantial effect on how gp140 is oligomerized via interactions between the $gp41_{ECTO}$ moieties, whereas the presence of the cysteine substitutions in gp120 and gp41 has little effect on these interactions. It is believed that this observation is central to understanding the relative instability of SOS gp140

oligomers, compared to those of the $gp140_{UNC}$ protein. It has not been determined, however, whether or not the intermolecular disulfide bond actually forms in SOS $gp140_{UNC}$; the simple method of DTT treatment to reduce this bond is inadequate, because the uncleaved peptide bond between the gp120 and $gp41_{ECTO}$ moieties still holds the two subunits together.

Discussion

The antigenic properties of SOS gp140, an HIV-1 envelope glycoprotein variant in which an intermolecular disulfide bond has been introduced to covalently link the gp120 and $gp41_{ECTO}$ subunits have been previously described (Binley, J.M., et al., J.Virol. 74:627-643 (2000); Sanders, R.W., et al., J.Virol. 74:5091-5100 (2000)). In the above report, it was demonstrated that the SOS gp140 protein, as contained in supernatants of transiently transfected 293T cells, was an antigenic mimic of virion-associated Env (Binley, J.M., et al., J.Virol. 74:627-643 (2000)). In that report, the methods employed were not sufficiently robust to conclusively determine the oligomeric state of unpurified 293T-derived SOS gp140 (Binley, J.M., et al., J.Virol. 74:627-643 (2000)). The present invention demonstrates that purified and unpurified CHO cell-derived SOS gp140 proteins also mimic native Env in terms of their patterns of antibody reactivity. However, unlike virus-associated Env, SOS gp140 is a monomeric protein.

Antigenicity and immunoelectron microscopy studies support a model for SOS gp140 in which the neutralizing face of gp120 is presented in a native conformation, but the non-neutralizing face is occluded by $gp41_{ECTO}$. The immunoelectron microscopy data suggest a model in which the $gp41_{ECTO}$ moiety of SOS gp140 occludes the non-neutralizing face of the gp120 subunit (Fig. 20). The evidence for this model is derived from several independent studies. In the first of these, SOS gp140 was examined in complex with combinations of anti-gp120 and anti-gp41 MAbs to defined epitopes (Fig. 18). The $gp41_{ECTO}$ subunit, as defined by the position of the anti-gp41 MAb 2F5, was located ~180B from the MAb

2G12 epitope and ~90B from the MAb IgG1b12 epitope, as is the non-neutralizing face. A second set of studies compared SOS gp140 and gp120 in complex with sCD4 and MAb 17b (Fig. 19). Here, a region of additional mass in the gp140 complex defined the presumptive $gp41_{ECTO}$. Its location was similarly adjacent to the non-neutralizing face of gp120. This model of the geometry of the gp120-gp41 interaction is consistent with previous models based on mutagenesis techniques and the mapping of MAb epitopes (Helseth, E., et al., J.Virol. 65:2119-2123 (1991); Moore, J.P. et al., J.Virol. 70 :1863-1872 (1996); Wyatt, R., et al., J.Virol. 71:9722-9731 (1997)). It also provides a basis for interpreting the patterns of MAb reactivity described above and discussed below.

For purposes of the present invention, the antigenicity of CHO-derived SOS gp140 was explored from a number of perspectives: (1) in comparison with $gp140_{UNC}$ and gp120; (2) before and after purification; (3) in an equilibrium-based assay (RIPA) *vs.* a kinetics-based assay (SPR). SOS gp140 proteins expressed in stably transfected CHO cells or transiently transfected 293T cells possessed qualitatively similar antigenic properties that were largely unaffected by purification. It was observed that most neutralizing anti-gp120 MAbs bound more strongly and more rapidly to SOS gp140 than to the gp120 or $gp140_{UNC}$ proteins, whereas the converse was true of non-neutralizing MAbs (Figs. 21, 22). These results were largely independent of the analytical methodology used (RIPA or SPR), or the purification state of the glycoproteins, and thus extend the earlier studies on the antigenicity of unpurified Env glycoproteins determined by RIPA (Binley, J.M., et al., J.Virol. 74:627-643 (2000)).

It is not obvious why neutralizing MAbs recognize monomeric SOS gp140 better than monomeric gp120. One possible reason relates to differences in the conformational freedom of the two glycoproteins. Monomeric gp120 has considerable conformational flexibility, such that "freezing" of the conformation by CD4 binding results in an unexpectedly large loss in entropy (Myszka, D.G., et al., Proc.Natl.Acad.Sci.U.S.A. 97:9026-9031 (2000)). Indeed, it has been suggested that reducing the conformational freedom of a gp120 immunogen may provide a means of generating broadly neutralizing antibodies, which generally recognize conformational epitopes (Myszka, D.G., et al., Proc.Natl.Acad.Sci.U.S.A. 97:9026-9031 (2000)). The presence of $gp41_{ECTO}$ may serve to minimize the conformational flexibility of the gp120 subunit of SOS gp140, stabilizing the protein in conformations recognized by neutralizing antibodies. However, the induction of 17b binding by sCD4 demonstrates that SOS gp140 is still capable of sampling multiple, relevant conformations.

Variations in conformational flexibility may also underlie the antigenic differences observed between the SOS gp140 and $gp140_{UNC}$ proteins. Other possible explanations include the effect that cleavage may have on the overall structure of Env, and differences in the oligomerization state of the two proteins. Standard biophysical techniques were used to demonstrate that the purified $HIV\text{-}1_{JR\text{-}FL}$ SOS gp140 glycoprotein is a monomer comprising one gp120 subunit disulfide-linked to $gp41_{ECTO}$. Since it is generally accepted that the gp41 subunits are responsible for Env trimerization (Caffrey, M., et al., EMBO J 17:4572-4584 (1998); Chan, D.C., et al, Cell 89:263-273 (1997); Lu, M., et al., Nat.Struct.Biol. 2:1075-1082 (1995); Tan, K., et al.,

Proc.Natl.Acad.Sci.U.S.A. 94:12303-12308 (1997); Weissenhorn, W., et al., Nature 387:426-430 (1997)), it was assumed that the gp41-gp41 interactions within the cleaved SOS gp140 glycoprotein are weak, and that this instability precludes the purification of cleaved trimers.

Also demonstrated herein is the application of a rapid, simple and high-resolution electrophoretic technique, BN-PAGE, for exploring the oligomeric state of HIV-1 envelope glycoproteins in unpurified as well as purified form. In this technique, the proteins of interest are combined with the dye coomassie blue, which binds to the exposed hydrophobic surfaces of proteins and usually enhances their solubility. In the presence of the dye, most proteins adopt a negative charge, migrate towards the anode in an electric field, and so can be sieved according to their Stokes' radius in a polyacrylamide gradient gel. Whereas traditional native PAGE methods are typically performed under alkaline conditions (pH 9.5), BN-PAGE uses a physiological pH (pH 7.5), which is more compatible with protein stability. It is demonstrated that a gp120/sCD4 complex and a variety of purified, oligomeric model proteins all remain associated during BN-PAGE analysis. When combined with Western blot detection, BN-PAGE can be used to determine the oligomeric state of HIV-1 envelope glycoproteins at all stages of purification. This high resolution technique can resolve monomeric, dimeric, trimeric and tetrameric forms of gp140.

As determined by BN-PAGE and other methods, the SOS gp140 protein was secreted in mostly monomeric form. In contrast, $gp140_{UNC}$ proteins, in which the peptide bond between gp120 and gp41 still attaches the two subunits, form oligomers that are significantly

more stable. Thus, it is shown herein that HIV-1_{JR-FL} $gp140_{UNC}$ comprises a mixture of dimers, trimers and tetramers, with dimers representing the major oligomeric form present under non-denaturing conditions. Although non-covalently associated oligomers constitute a significant percentage of the $gp140_{UNC}$ preparation, half or more of the material consists of disulfide-linked and presumably misfolded material (Owens, R.J. et al., Virology 179:827-833 (1999)). Others have made similar observations with uncleaved gp140 proteins from other HIV-1 strains, and from SIV (Chen, B., et al. J.Biol.Chem. 275:34946-34953 (2000); Earl, P.L., et al., J.Virol. 71:2674-2684 (1997); Earl, P.L., et al., J.Virol. 68:3015-3026 (1994); Earl, P.L., et al., Proc.Nat.Acad.Sci.USA 87:648-652 (1990); Earl, P.L., et al., J.Virol. 75:645-653 (2001); Edinger, A.L., et al., J.Virol. 74:7922-7935 (2000); Farzan, M., et al., J.Virol. 72:7620-7625 (1998); Hoffman, T.L., et al., Proc.Natl.Acad.Sci.U.S.A. 97:11215-11220; Owens, R.J. et al., Virology 179:827-833 (1999); Richardson, T.M.J., et al., J.Virol. 70:753-762 (1996); Stamatatos, L., et al., AIDS Res.Hum.Retroviruses 16:981-994 (2000); Staropoli, I., et al., J.Biol.Chem. 275:35137-35145 (2000); Yang, X., et al., J.Virol. 74:5716-5725 (2000); Yang, X., et al., J.Virol. 74:4746-4754 (2000); Yang, X., et al., J.Virol. 75:1165-1171 (2001)).

The question then arises as to why the SOS gp140 protein is a monomer, but the uncleaved proteins are oligomeric. It is believed that the cleavage of the gp120-gp41 peptide bond alters the overall conformation of the envelope glycoprotein complex, rendering it fusion-competent but also destabilizing the association between the gp41 subunits. Support for this argument

is provided by the evidence that the SOS $gp140_{UNC}$ protein behaves identically to the $gp140_{UNC}$ protein, but very differently from the SOS gp140 protein; cleavage is clearly more important than the engineered, intermolecular disulfide bind in determining the oligomeric stability of gp140 proteins. It may be hypothesized that destabilization of gp41-gp41 interactions might be necessary for gp41-mediated fusion to occur efficiently upon activation of the Env complex by gp120-receptor interactions. Moreover, having cleavage/activation take place late in the synthetic process minimizes the risk of fusion events occurring prematurely, *i.e.*, during intracellular transport of the envelope glycoprotein complex.

Taken together, the antigenic and biophysical data of SOS gp140, gp120 and $gp140_{UNC}$ suggest that SOS gp140 represents an improved yet clearly imperfect mimic of native Env. It is perhaps surprising that an SOS gp140 monomer mimics virus-associated Env in its reactivity with a diverse panel of MAbs. Immunochemical studies and the X-ray crystal structure of the gp120 core in complex with CD4 and MAb 17b have together defined the surface of gp120 in terms of neutralizing, non-neutralizing and silent faces (Kwong, P.D., et al., Nature 393:648-659 (1998); Wyatt, R., et al., Nature 393:705-711 (1998)). The data presented here and elsewhere (Binley, J.M., et al., J.Virol. 74:627-643 (2000)) demonstrate the neutralizing face is readily accessible on SOS gp140, whereas the non-neutralizing face is not. There are still no immunologic ways to probe the exposure of the silent face of gp120 (Moore, J.P., et al., J. Virol. 70:1863-1872 (1996)). A source of purified SOS gp140 glycoprotein, as described herein, will facilitate further studies of the antigenic structure of SOS gp140 in comparison with that of native Env.

It is not believed that $gp140_{UNC}$ proteins mimic the structure of the native, fusion-competent envelope glycoprotein complex on virions, based on their exposure of non-neutralizing epitopes in both gp120 and gp41 that are not accessible on the surface of native envelope glycoprotein complexes (Binley, J.M., et al., J.Virol. 74:627-643 (2000); Sattentau, Q.J., et al., Virology 206:713-717 (1995)). Neutralization epitopes overlapping the CD4 binding site are poorly presented on $HIV\text{-}1_{BH8}$ $gp140_{UNC}$ relative to virus-associated Env (Parren, P.W.H.I., et al., J.Virol. 70:9046-9050 (1996)), and only one CD4 molecule can bind to the SIV_{mac32H} $gp140_{UNC}$ protein. The lack of correlation between the binding of MAbs to uncleaved envelope glycoprotein complexes on the surface of Env-transfected cells and neutralization of the corresponding viruses again argues that uncleaved complexes have an abnormal configuration (York, J., et al., J.Virol. 75:2741-2752 (2001)). However, in the absence of definitive and comparative structural information on native and uncleaved Env complexes, this is an unresolved point. At present it is not possible to predict what antigenic structures will elicit a desired immune response; that can only be defined empirically, and it may be that one or more uncleaved forms of Env will be effective immunogens even if they do not properly mimic the structure of the native Env complex.

Given that SOS gp140 is monomeric, the question arises as to what can be done to further stabilize the structure of fully cleaved, envelope glycoprotein complexes? The immunoelectron microscopy data of the 2G12/SOS gp140 complex suggest that appropriately directed antibodies could strengthen weak oligomeric interactions. The immunogenicity of such complexes may be worth

testing, although a bivalent MAb might be expected to promote formation of Env dimers rather than trimers. An attempt has been made to combine the SOS gp140 disulfide bond stabilization strategy with one in which the gp41 subunits were also stabilized by an intermolecular disulfide bond B. This attempt was unsuccessful, in that the mutated protein was poorly expressed and could not be cleaved into gp120 and gp41 subunits, even in the presence of co-transfected furin. Similarly, adding GCN-4 domains onto the C-terminus of gp41 hindered the proper cleavage of gp140 into gp120 and gp41 furin.

Fortuitously, it has been found that variable-loop-deleted forms of HIV-1_{JR-FL} SOS gp140 form more stable oligomers than their full-length counterparts. Thus, the SOS gp140 proteins lacking either the V1 or V2 variable loops contain a greater proportion of oligomers than the full-length protein, and the V1V2 double loop-deletant is expressed primarily as noncovalently-associated trimers. One hypothesis is that the extended and extensively glycosylated variable loops sterically impede the formation of stable gp41-gp41 interactions in the context of the full-length SOS gp140 protein. Indeed, using the crystal structure of the gp120/CD4/17b complex, Kwong *et al.* have developed a model of oligomeric gp120 that places V1V2 sequences at the trimer interface (Kwong, P.D., et al., J.Virol. 74:1961-1972 (2000)). The variable-loop-deleted SOS gp140 proteins may therefore represent proteolytically mature HIV-1 envelope glycoproteins that can perhaps eventually be produced and purified as oligomers.

CLAIMS

What is claimed:

1. An isolated nucleic acid which comprises a nucleotide segment having a sequence encoding a complex comprising a viral surface protein and a corresponding viral transmembrane protein wherein the complex contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and the viral transmembrane protein.

2. The isolated nucleic acid of claim 1, wherein the virus is a lentivirus.

3. The isolated nucleic acid of claim 1, wherein the virus is the human immunodeficiency virus.

4. The isolated nucleic acid of claim 3, wherein the human immunodeficiency virus is a primary isolate.

5. The isolated nucleic acid of claim 3, wherein the human immunodeficiency virus is HIV-1_{JR-FL}, HIV-1_{DH123}, HIV-1_{Gun-1}, HIV-$1_{89.6}$, or HIV-1_{HXB2}.

6. The isolated nucleic acid of claim 3, wherein the viral surface protein is gp120 or a modified form of gp120, wherein the modification alters the immunogenicity of the molecule relative to wild type gp120.

7. The isolated nucleic acid of claim 6, wherein the modified

gp120 molecule is characterized by the absence of one or more variable loops present in wild type gp120.

8. The isolated nucleic acid of claim 7, wherein the variable loop comprises V1, V2, or V3.

9. The isolated nucleic acid of any one of claims 6- 8, wherein the modified gp120 molecule is characterized by the absence or presence of one or more canonical glycosylation sites present absent or absent in wild type gp120.

10. The isolated nucleic acid of claim 9, wherein one or more canonical glycosylation sites are absent from the V1V2 region of the gp120 molecule.

11. The isolated nucleic acid of any one of claims 3-10, wherein the transmembrane protein is gp41 or a modified form of gp41, wherein the modification alters the immunogenicity of the molecule relative to wildtype gp41.

12. The isolated nucleic acid of claim 11, wherein the transmembrane protein is the gp41 ectodomain.

13. The isolated nucleic acid of claim 11 or 12, wherein the transmembrane protein is modified by the absence or presence of one or more canonical glycosylation sites absent or present in the wild type gp120.

14. The isolated nucleic acid of any one of claims 1-13, wherein the stabilization of the complex is achieved by one or more cysteine-cysteine bonds that are formed between the

surface and transmembrane proteins and that are not present in the corresponding wildtype complex.

15. The isolated nucleic acid of claim 14, wherein one or more amino acids which are adjacent to or which contain an atom within 5 angstroms of an introduced cysteine are mutated to a noncysteine residue.

16. The isolated nucleic acid of claim 14 or 15, wherein one or more cysteines in gp120 or modified form of gp120 are disulfide linked to one or more cysteines in gp41 or modified form of gp41..

17. The isolated nucleic acid of claim 16, wherein a cysteine in the C5 region of gp120 or modified form of gp120 is disulfide linked to a cysteine in the ectodomain of gp41.

18. The isolated nucleic acid of claim 16, wherein the disulfide bond is formed between a cysteine introduced by an A492C mutation in gp120 and a T596C mutation in gp41.

19. The isolated nucleic acid molecule of claim 1 which is cDNA.

20. The isolated nucleic acid molecule of claim 1 which is genomic DNA.

21. The isolated nucleic acid molecule of claim 1 which is RNA.

22. A replicable vector comprising the nucleic acid of claim 1.

23. A plasmid, cosmid, λ phage or YAC containing the nucleic acid of claim 1.

24. The plasmid of claim 23 designated PPI4.

25. A host cell containing the vector of claim 22.

26. The cell of claim 25 which is a eukaryotic cell.

27. The cell of claim 25 which is a bacterial cell.

28. A vaccine which comprises the isolated nucleic acid of claim 1.

29. A vaccine which comprises a therapeutically effective amount of the nucleic acid of claim 1.

30. A vaccine which comprises a therapeutically effective amount of the protein encoded by the nucleic acid of claim 1.

31. A method of treating a viral disease which comprises immunizing a virally infected subject with the vaccine of claim 29 or 30 or a combination thereof, thereby treating the subject.

32. A vaccine which comprises a prophylactically effective amount of the nucleic acid of claim 1.

33. A vaccine which comprises a prophylactically effective amount of the protein encoded by the nucleic acid of claim 1.

34. A method of reducing the likelihood of a subject becoming infected with a virus comprising administering the vaccine of claim 32 or 33 or a combination thereof, thereby reducing the likelihood of the subject becoming infected with the virus.

35. A vaccine comprising the nucleic acid of any one of claims 3-18.

36. A vaccine which comprises a therapeutically effective amount of the nucleic acid of any one of claims 3-18.

37. A vaccine which comprises a therapeutically effective amount of the protein encoded by the nucleic acid of any one of claims 3-18.

38. A method of treating an HIV-1 infected subject which comprises immunizing the subject with the vaccine of claim 36 or 37 or a combination thereof, thereby treating the subject.

39. A vaccine which comprises a prophylactically effective amount of the nucleic acid of any one of claims 3-18.

40. A vaccine which comprises a prophylactically effective amount of the protein encoded by the nucleic acid of any

one of claims 3-18.

41. A method of reducing the likelihood of a subject becoming infected with HIV-1 comprising administering the vaccine of claim 39 or 40 or a combination thereof, thereby reducing the likelihood of the subject becoming infected with HIV-1.

42. The vaccine of claim 35, wherein the vaccine comprises a recombinant subunit protein, a DNA plasmid, a replicating viral vector, a non-replicating viral vector, or a combination thereof.

43. A method of reducing the severity of HIV-1 disease in a subject comprising administering the vaccine of claim 39 or 40 or a combination thereof, prior to exposure of the subject to HIV-1, thereby reducing the severity of HIV-1 disease or AIDS in the subject upon subsequent exposure to HIV-1.

44. A viral envelope protein comprising a viral surface protein and a corresponding viral transmembrane protein wherein the viral envelope protein contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and transmembrane protein.

45. A complex comprising a viral surface protein and a viral transmembrane protein, wherein the complex contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and transmembrane protein.

46. A mutant HIV-1 envelope protein which is encoded by the nucleic acid of any one of claims 3-18.

47. The protein of claim 44 or complex of claim 45 which is linked to at least one other protein or protein fragment to form a fusion protein.

48. A purified protein of any one of claims 44-46.

49. A vaccine which comprises a therapeutically effective amount of the protein of claim 44 or the complex of claim 45.

50. A vaccine which comprises a prophylactically effective amount of the protein of claim 44 or the complex of claim 45.

51. A method of stimulating or enhancing in a subject production of antibodies which recognize the protein of claim 44 or complex of claim 45.

52. An antibody, antibody chain, fragment or derivative thereof isolated or identified using the viral envelope protein encoded by the recombinant nucleic acid of claim 1.

53. The antibody of claim 52, wherein the antibody is of the IgM, IgA, IgE or IgG class or subclasses thereof.

54. The antibody fragment of claim 52 which includes but is not limited to Fab, Fab' $(Fab')_2$, Fv and single chain

antibodies.

55. The isolated antibody light chain of the antibody of claim 52, or fragment or oligomer thereof.

56. The isolated antibody heavy chain of the antibody of claim 52, or fragment or oligomer thereof.

57. One or more complementarity determining regions of the antibody of claim 52.

58. The antibody of claim 52 which is derivatized such as by the addition of a fluorescent moiety, a radionuclide, an enzyme, a toxin, or an affinity ligand such as biotin.

59. The antibody of claim 52 wherein the antibody is a human antibody.

60. The antibody of claim 52 or 59, wherein the antibody is a monoclonal antibody.

61. The antibody of claim 52, wherein the antibody is a humanized antibody.

62. The antibody of claim 52 or any one of claims 59-61, wherein the viral envelope protein is derived from HIV-1.

63. An isolated nucleic acid molecule encoding the antibody of claim 52 or any one of claims 59-61, wherein the nucleic acid molecule is RNA, genomic DNA or cDNA.

64. The isolated nucleic acid of claim 63, wherein the viral envelope protein is derived from HIV-1.

65. An agent capable of inhibiting the binding of the antibody of claim 52.

66. A method of reducing the likelihood of an HIV-1-exposed subject from becoming infected with HIV-1 comprising administering the antibody of claim 62 or the isolated nucleic acid of claim 64, thereby reducing the likelihood of the HIV-1 exposed subject from becoming infected with HIV-1.

67. A method of treating a subject infected with HIV-1 comprising administering the antibody of claim 62 or the isolated nucleic acid of claim 64, thereby treating the subject.

68. An agent capable of binding the mutant viral envelope protein encoded by the recombinant nucleic acid molecule of claim 1.

69. The agent of claim 68 which inhibits viral infection.

70. The agent of claim 69, wherein the viral envelope protein is derived from HIV-1.

71. A method for determining whether a compound is capable of inhibiting a viral infection comprising:
(A) contacting an appropriate concentration of the

compound with the mutant viral envelope protein encoded by the nucleic acid of claim 1 under conditions permitting binding of the compound to said protein;

(B) contacting the resulting complex with a reporter molecule under conditions that permit binding of the reporter molecule to the mutant viral envelope protein in the absence of the compound;

(C) measuring the amount of bound reporter molecule; and

(D) comparing the amount of bound reporter molecule in step (c) with the amount determined in the absence of the compound, a decrease in the amount indicating that the compound is capable of inhibiting infection by the virus.

72. The method of claim 71, wherein the reporter molecule is an antibody or derivative thereof.

73. The method of claim 71, wherein the reporter molecule comprises one or more host cell viral receptors or molecular mimics thereof.

74. A method for determining whether a compound is capable of inhibiting a viral infection which comprises:

(a) contacting an appropriate concentration of the compound with a host cell viral receptor or molecular mimic thereof under conditions that permit binding of the compound and receptor or receptor mimic in the absence of the compound;

(b) contacting the resulting complex with the mutant viral envelope protein encoded by the recombinant nucleic

acid of claim 1 under conditions that permit binding of the envelope protein and receptor or receptor mimic in the absence of the compound;

(c) measuring the amount of binding of envelope protein to receptor or receptor mimic;

(d) comparing the amount of binding determined in step (c) with the amount determined in the absence of the compound, a decrease in the amount indicating that the compound is capable of inhibiting infection by the virus.

75. The method of any one of claims 71-74 wherein the virus is HIV-1.

76. The method of claim 71 or 72, wherein the host cell viral receptor is CD4, CCR5, CXCR4 or combinations or molecular mimics thereof.

77. The method of any one of claims 71-76, wherein the compound was not previously known.

78. A compound determined to be capable of inhibiting a viral infection by the method of any one of claims 71-76.

79. A pharmaceutical composition comprising an amount of the compound effective to inhibit viral infection determined by the method of any one of claims 71-76 to be capable of inhibiting viral infection and a pharmaceutically acceptable carrier.

80. The pharmaceutical composition of claim 79, wherein the viral infection is HIV-1 infection.

81. A viral envelope protein comprising a viral surface protein and a corresponding viral transmembrane protein wherein the viral envelope protein contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and transmembrane protein, wherein the surface protein and transmembrane protein are encoded by different nucleic acids.

82. A complex comprising a viral surface protein and a corresponding viral transmembrane protein of a viral envelope protein wherein the viral envelope protein contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and transmembrane protein, wherein the surface protein and transmembrane protein are encoded by different nucleic acids.

83. An antibody which binds to the protein of claim 44 or the complex of claim 45 but does not cross react with the individual monomeric surface protein or the individual monomeric transmembrane protein.

84. The antibody of claim 83 capable of binding to the HIV-1 virus.

85. A virus-like particle which comprises the complex of claim

45.

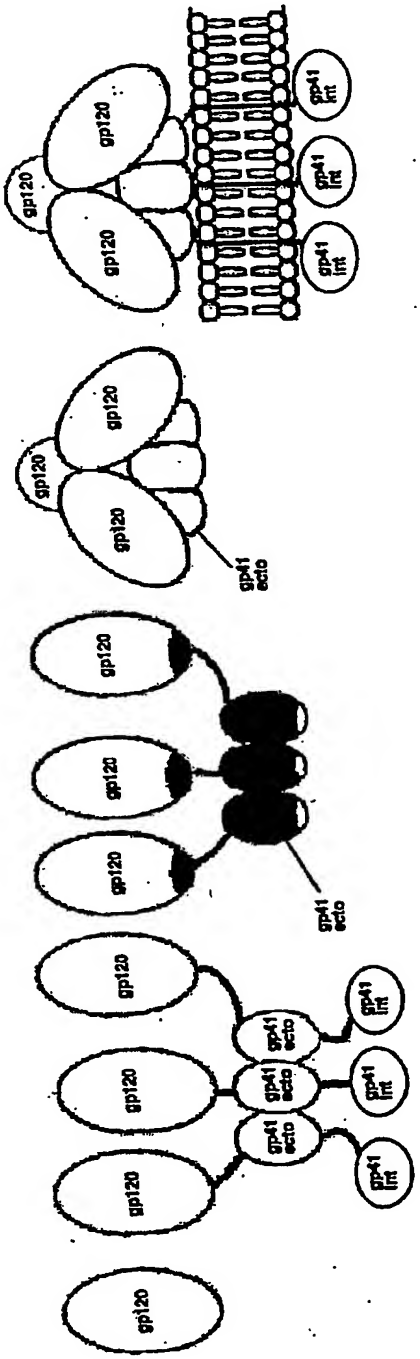
86. The virus-like particle of claim 85, further comprising an immunodeficiency virus gag protein.

STABILIZED VIRAL ENVELOPE PROTEINS AND USES THEREOF

Abstract of the Disclosure

This invention provides an isolated nucleic acid which comprises a nucleotide segment having a sequence encoding a viral envelope protein comprising a viral surface protein and a corresponding viral transmembrane protein wherein the viral envelope protein contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and transmembrane protein. This invention also provides a viral envelope protein comprising a viral surface protein and a corresponding viral transmembrane protein wherein the viral envelope protein contains one or more mutations in amino acid sequence that enhance the stability of the complex formed between the viral surface protein and transmembrane protein. This invention further provides methods of treating HIV-1 infection.

Figure 1


gp120
gp120
gp120
gp41 int
gp41 int
gp41 int
gp120
gp120
gp120
gp41 ecto
gp120
gp120
gp120
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gp41 ecto
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gp41 int

Figure 2

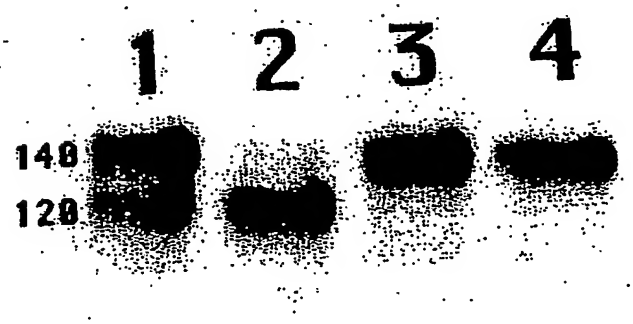


Figure 3A

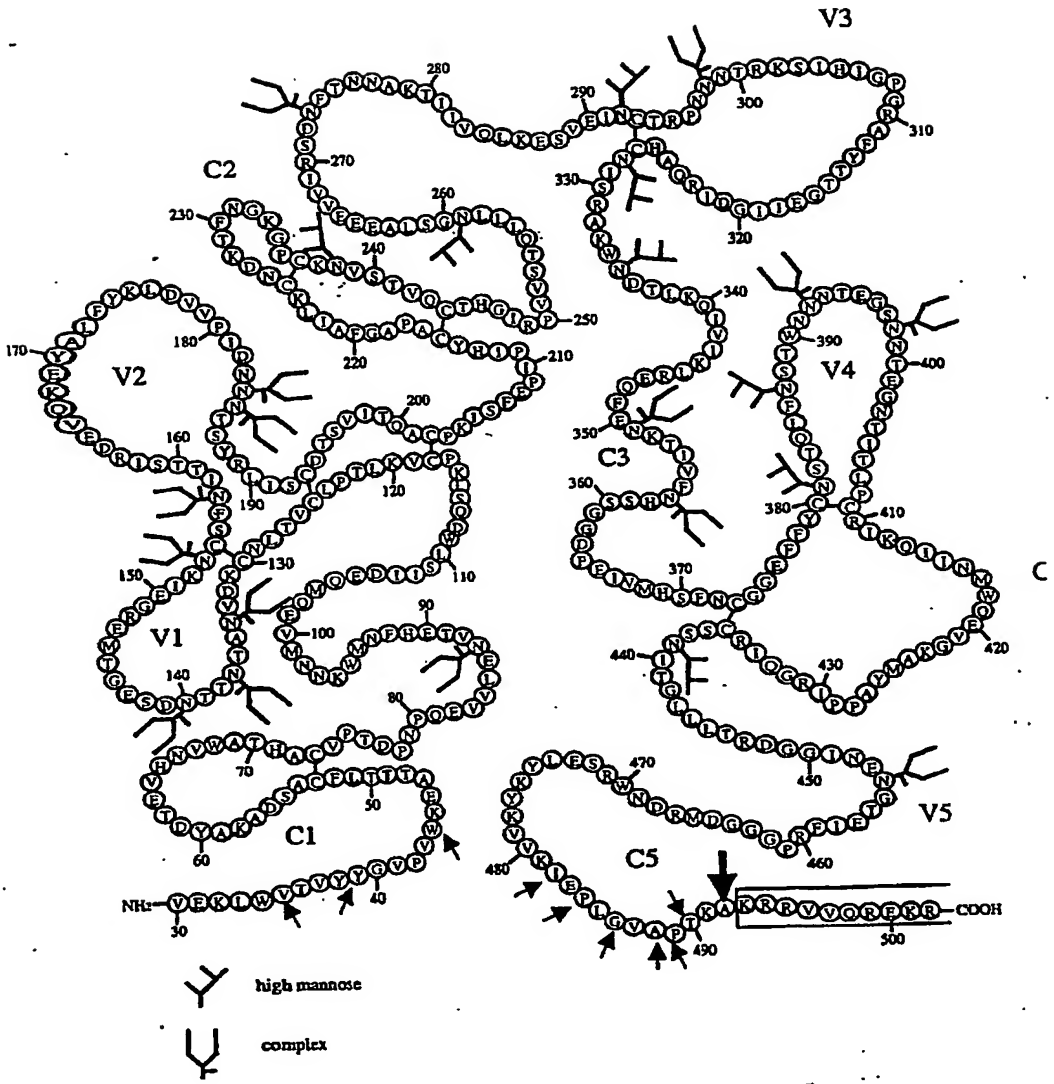
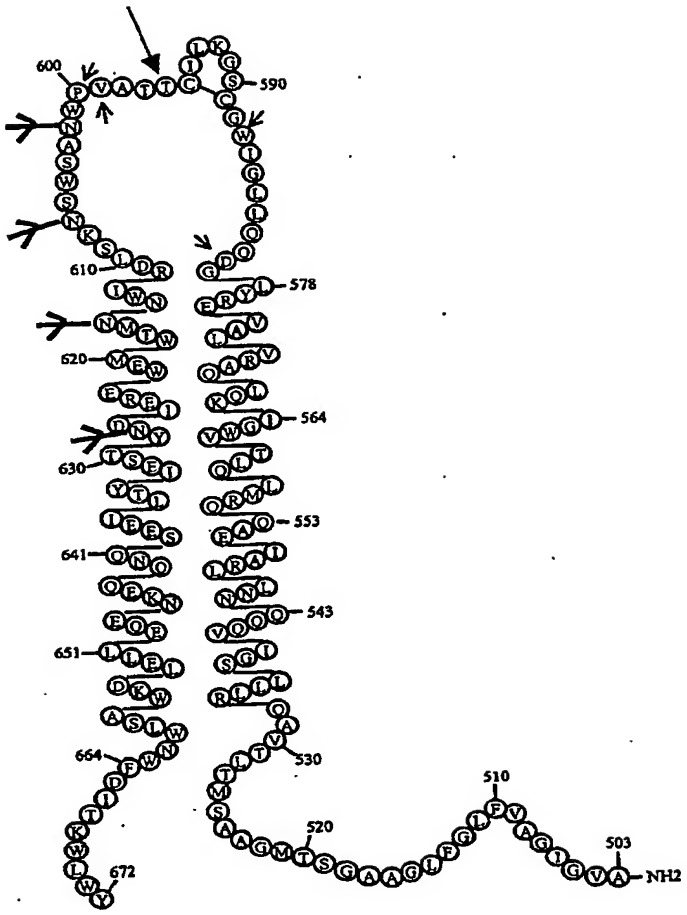


Figure 3B


600
590
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578
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564
630
553
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543
651
530
654
510
520
503
672
NH2

Figure 4

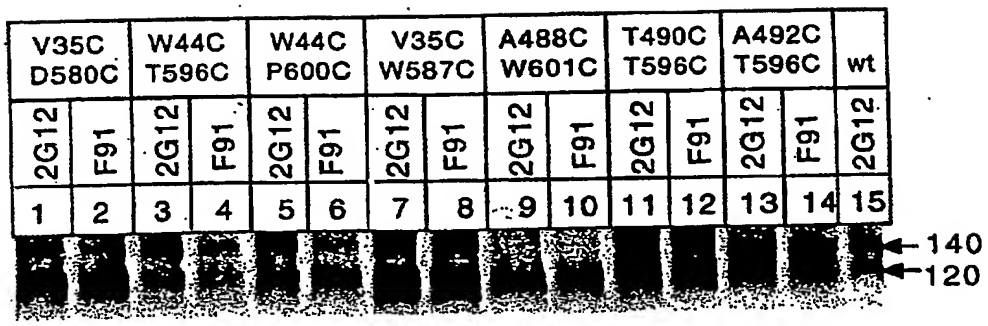


Figure 5

gp41

	D580C	W587C	T596C	V599C	P600C	W601C
V35C	0.45	0.40	0.35	0.30	0.40	0.30
Y39C	0.35	0.30	0.60	0.45	0.45	N.D.
W44C	0.45	0.45	0.65	0.50	0.65	0.45

gp120 C1

P484C	0.35	0.30	0.45	0	0	0
G486C	0	0	0.25	0.20	0.30	0
A488C	0	0	0.05	0	0	0
P489C	0	0.10	0.30	0.15	0.05	0
T490C	0	0.15	0.55	0.25	0.25	0.10
A492C	0.05	0	0.75	0.50	0.10	0.25

gp120 C5

Figure 6

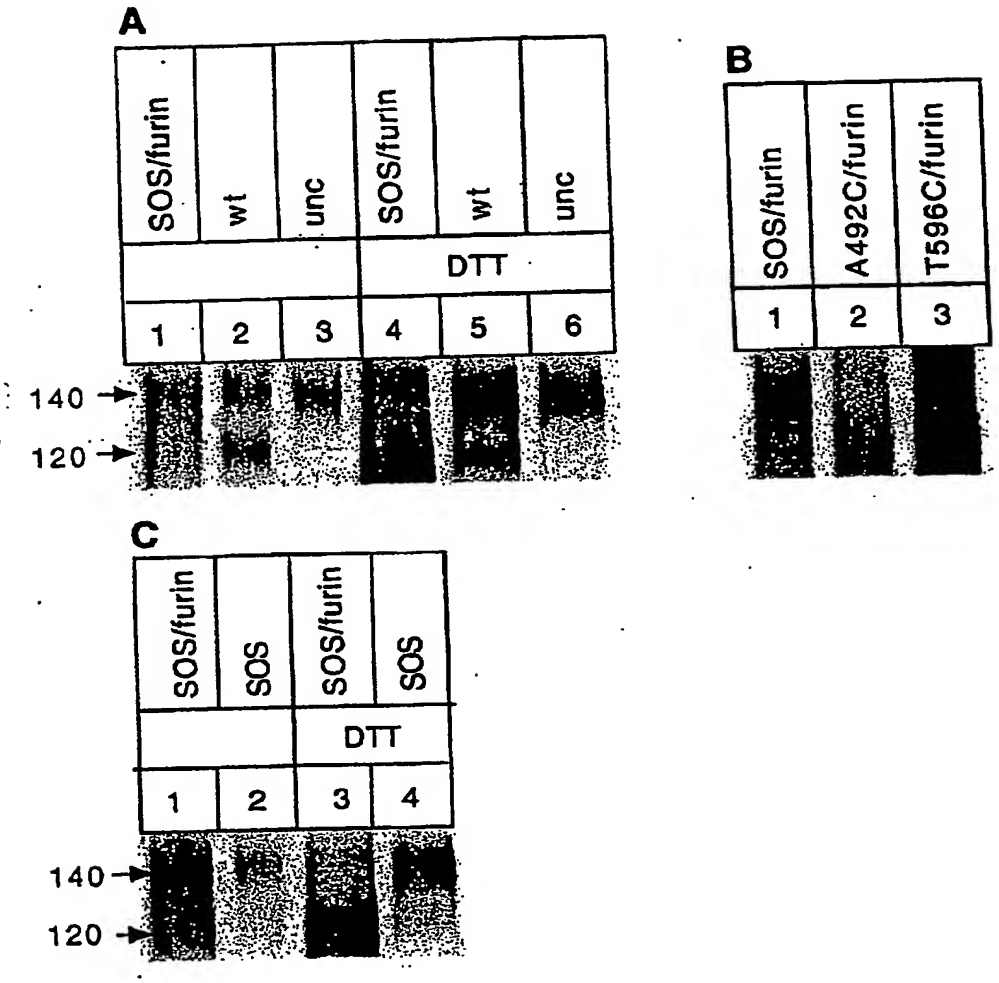


Figure 7

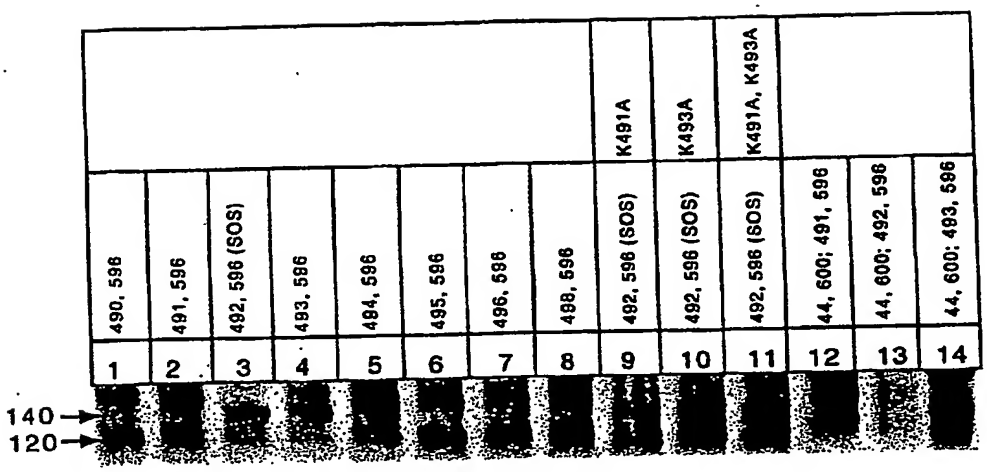
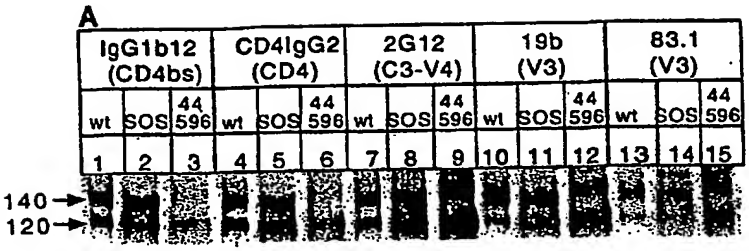


Figure 8A



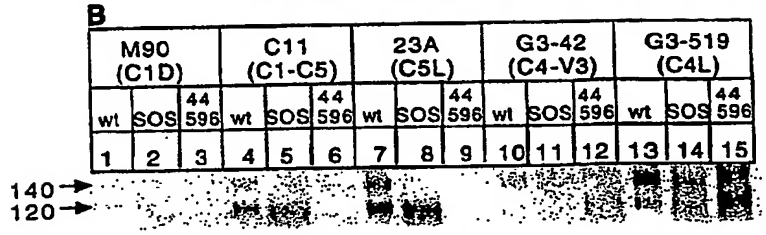


Figure 8B

Figure 8C

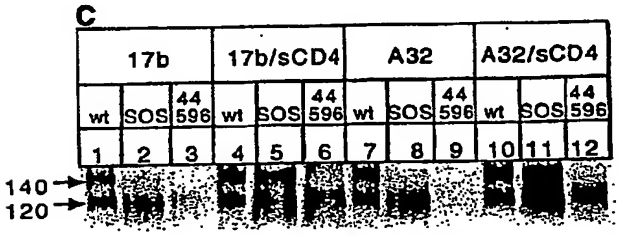


Figure 8D

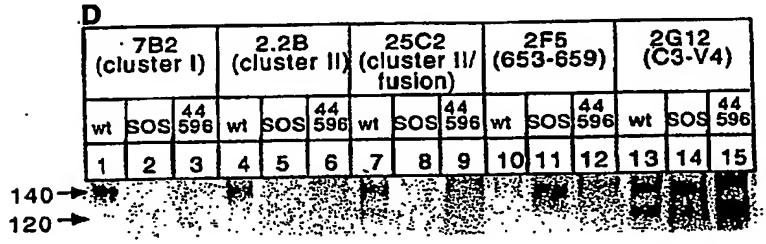


Figure 8E

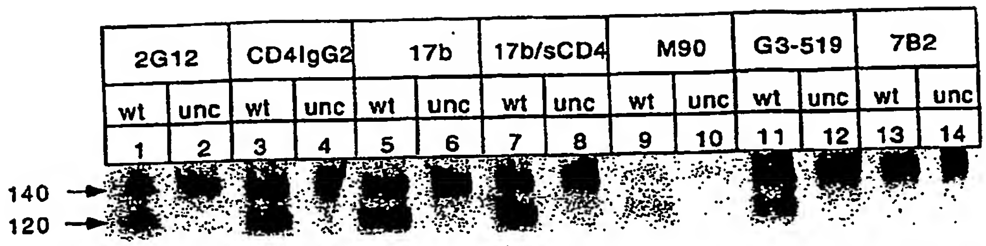


Figure 9

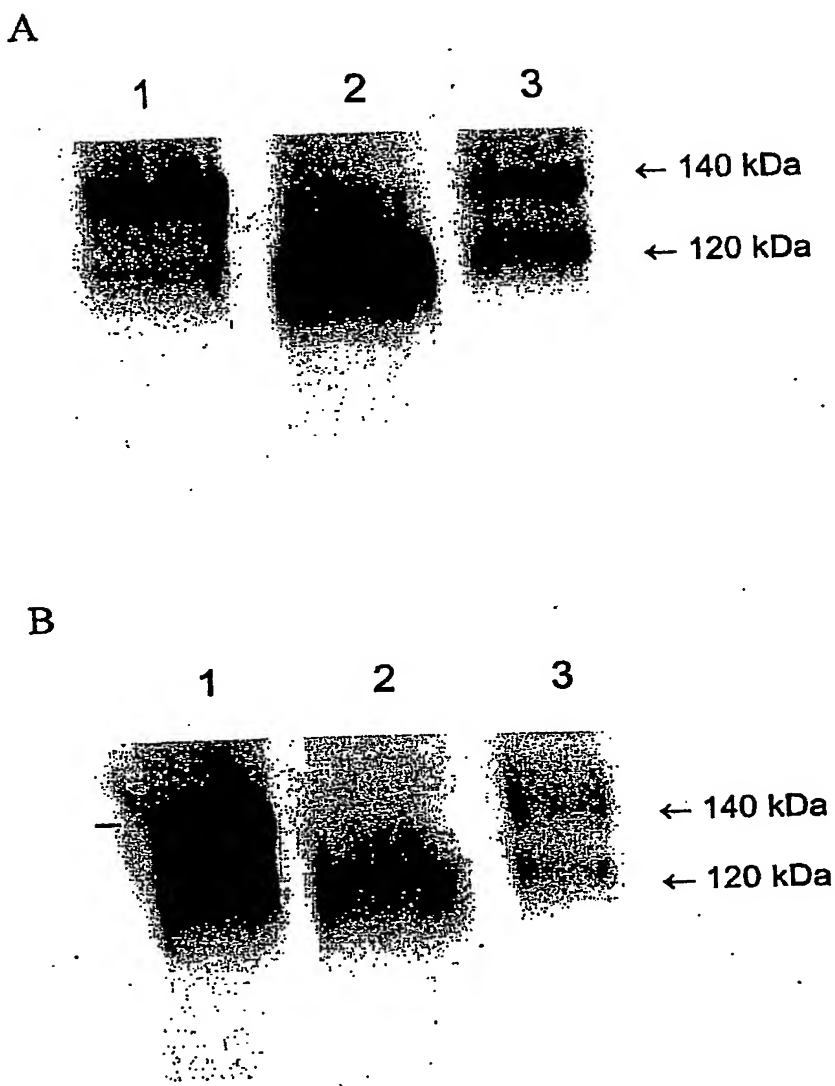


Figure 10

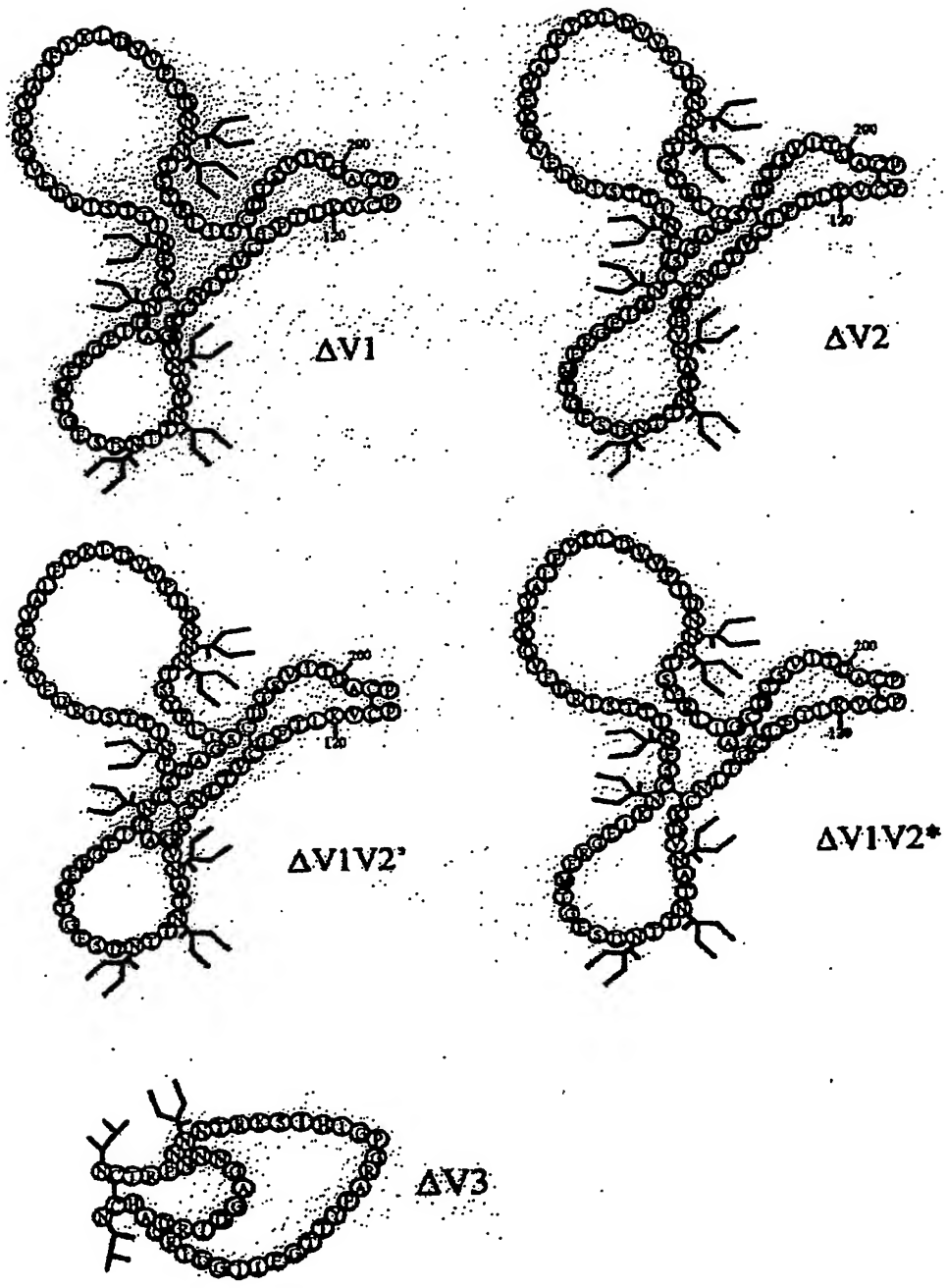
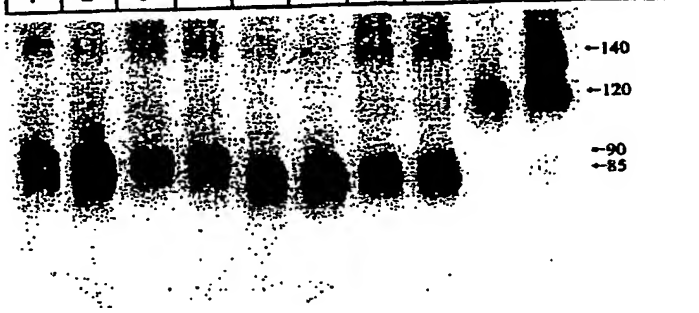


Figure 11

A

wt ΔV1V2*V3		CC ΔV1V2*V3		wt ΔV1V2*V3 N357Q N398Q		CC ΔV1V2*V3 N357Q N398Q		wt		envelope protein
2G12	F91	2G12	F91	2G12	F91	2G12	F91	F91	F91	antibody
1	2	3	4	5	6	7	8	9	10	lane



B

wt	ΔV1		ΔV2		ΔV3		ΔV1V2'		ΔV1V2*		ΔV1V2*V3		protein
CC		CC		CC		CC		CC		CC		CC	cysteines
1	2	3	4	5	6	7	8	9	10	11	12	13	lane

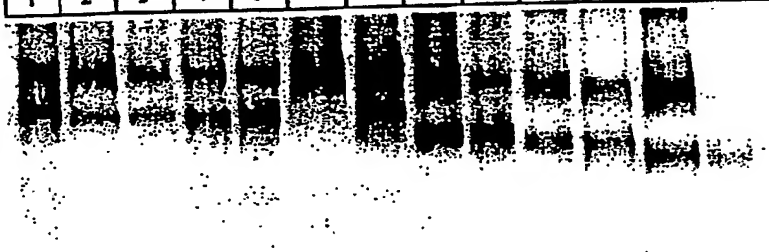


Figure 12

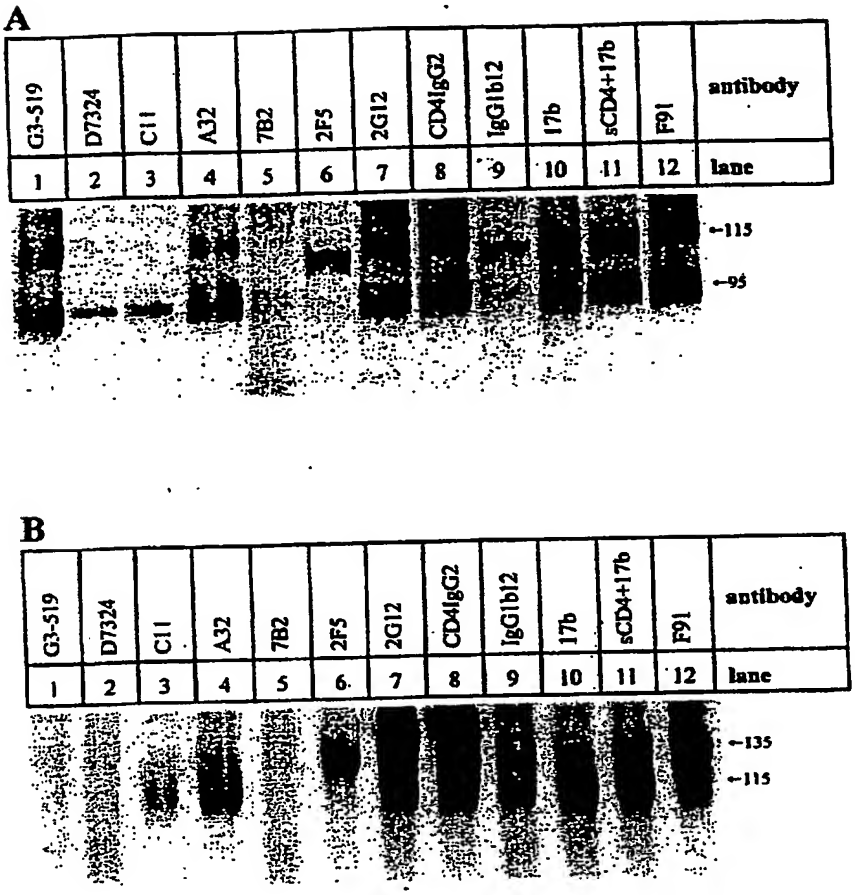


Figure 13

HIV-1_{JR-FL} SOS gp140

(a)

```
1     GTAGAAAAGTTGTGGGTCACAGTCTATTATGGGGTACCTGTGTGGAAAGA
51    AGCAACCACCACTCTATTTTGTGCATCAGATGCTAAAGCATATGATACAG
101   AGGTACATAATGTTTGGGCCACACATGCCTGTGTACCCACAGACCCCAAC
151   CCACAAGAAGTAGTATTGGAAAATGTAACAGAACATTTTAACATGTGGAA
201   AAATAACATGGTAGAACAGATGCAGGAGGATATAATCAGTTTATGGGATC
251   AAAGCCTAAAGCCATGTGTAAAATTAACCCCACTCTGTGTTACTTTAAAT
301   TGCAAGGATGTGAATGCTACTAATACCACTAATGATAGCGAGGGAACGAT
351   GGAGAGAGGAGAAATAAAAAACTGCTCTTTCAATATCACCACAAGCATAA
401   GAGATGAGGTGCAGAAAGAATATGCTCTTTTTTATAAACTTGATGTAGTA
451   CCAATAGATAATAATAATACCAGCTATAGGTTGATAAGTTGTGACACCTC
501   AGTCATTACACAGGCCTGTCCAAAGATATCCTTTGAGCCAATTCCCATAC
551   ATTATTGTGCCCCGGCTGGTTTTGCGATTCTAAAGTGTAATGATAAGACG
601   TTCAATGGAAAAGGACCATGTAAAAATGTCAGCACAGTACAATGTACACA
651   TGGAATTAGGCCAGTAGTATCAACTCAACTGCTGCTAAATGGCAGTCTAG
701   CAGAAGAAGAGGTAGTAATTAGATCTGACAATTTCACGAACAATGCTAAA
751   ACCATAATAGTACAGCTGAAAGAATCTGTAGAAATTAATTGTACAAGACC
801   CAACAACAATACAAGAAAAAGTATACATATAGGACCAGGGAGAGCATTTT
851   ATACTACAGGAGAAATAATAGGAGATATAAGACAAGCACATTGTAACATT
901   AGTAGAGCAAAATGGAATGACACTTTAAAACAGATAGTTATAAAATTAAG
951   AGAACAATTTGAGAATAAAACAATAGTCTTTAATCACTCCTCAGGAGGGG
1001  ACCCAGAAATTGTAATGCACAGTTTTAATTGTGAAGGAGAATTTTTCTAC
1051  TGTAATTCAACACAACTGTTTAATAGTACTTGGAATAATAATACTGAAGG
1101  GTCAAATAACACTGAAGGAAATACTATCACACTCCCATGCAGAATAAAAC
1151  AAATTATAAACATGTGGCAGGAAGTAGGAAAAGCAATGTATGCCCCTCCC
1201  ATCAGAGGACAAATTAGATGTTCATCAAATATTACAGGGCTGCTATTAAC
1251  AAGAGATGGTGGTATTAATGAGAATGGGACCGAGATCTTCAGACCTGGAG
1301  GAGGAGATATGAGGGACAATTGGAGAAGTGAATTCTATAAATATAAAGTA
1351  GTAAAAATTGAACCATTAGGAGTAGCACCCACCAAGTGCAAGAGAAGAGT
1401  GGTGCAAAGAGAAAAAAGAGCAGTGGGAATAGGAGCTGTGTTCCTTGGGT
1451  TCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATGACACTGACG
1501  GTACAGGCCAGACTATTATTGTCTGGTATAGTGCAACAGCAGAACAATTT
1551  GCTGAGGGCTATTGAGGCGCAACAGCGTATGTTGCAACTCACAGTCTGGG
1601  GCATCAAGCAGCTCCAGGCAAGAGTCCTGGCTGTGGAAAGATACCTAGGG
1651  GATCAACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAACTCATTTGCTG
1701  CACTGCTGTGCCTTGGAATGCTAGTTGGAGTAATAAATCTCTAGATAGGA
1751  TTTGGAATAACATGACCTGGATGGAGTGGGAAAGAGAAATTGACAATTAC
1801  ACAAGCGAAATATACACACTAATTGAAGAATCGCAGAACCAACAAGAAAA
1851  GAATGAACAAGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATT
1901  GGTTTGACATAACAAACTGGCTGTGGTAT
```

Figure 13

(b)

```
30    VEKLWVTVYY GVPVWKEATT TLFCASDAKA YDTEVHNVWA THACVPTDPN
80    PQEVVLENVT EHFNMWKNNM VEQMQEDIIS LWDQSLKPCV KLTPLCVTLN
130   CKDVNATNTT NDSEGTMERG EIKNCSFNIT TSIRDEVQKE YALFYKLDVV
180   PIDNNNTSYR LISCDTSVIT QACPKISFEP IPIHYCAPAG FAILKCNDKT
230   FNGKGPCKNV STVQCTHGIR PVVSTQLLLN GSLAEEEVVI RSDNFTNNAK
280   TIIVQLKESV EINCTRPNNN TRKSIHIGPG RAFYTTGEII GDIRQAHCNI
330   SRAKWNDTLK QIVIKLREQF ENKTIVFNHS SGGDPEIVMH SFNCEGEFFY
380   CNSTQLFNST WNNNTEGSNN TEGNTITLPC RIKQIINMWQ EVGKAMYAPP
430   IRGQIRCSSN ITGLLLTRDG GINENGTEIF RPGGGDMRDN WRSEFYKYKV
480   VKIEPLGVAP TKCKRRVVQR EKRAVGIGAV FLGFLGAAGS TMGAASMTLT
530   VQARLLLSGI VQQQNNLLRA IEAQQRMLQL TVWGIKQLQA RVLAVERYLG
580   DQQLLGIWGC SGKLICCTAV PWNASWSNKS LDRIWNNMTW MEWEREIDNY
630   TSEIYTLIEE SQNQQEKNEQ ELLELDKWAS LWNWFDITNW LWY
```

Figure 14

HIV-1_{JR-FL} ΔV1V2* SOS gp140

(a)

```
1     GTAGAAAAGTTGTGGGTCACAGTCTATTATGGGGTACCTGTGTGGAAAGA
51    AGCAACCACCACTCTATTTTGTGCATCAGATGCTAAAGCATATGATACAG
101   AGGTACATAATGTTTGGGCCACACATGCCTGTGTACCCACAGACCCCAAC
151   CCACAAGAAGTAGTATTGGAAAATGTAACAGAACATTTTAACATGTGGAA
201   AAATAACATGGTAGAACAGATGCAGGAGGATATAATCAGTTTATGGGATC
251   AAAGCCTAAAGCCATGTGTAAAATTAACCCCACTCTGTGGTGCAGGATGT
301   GACACCTCAGTCATTACACAGGCCTGTCCAAAGATATCCTTTGAGCCAAT
351   TCCCATACATTATTGTGCCCCGGCTGGTTTTGCGATTCTAAAGTGTAATG
401   ATAAGACGTTCAATGGAAAAGGACCATGTAAAAATGTCAGCACAGTACAA
451   TGTACACATGGAATTAGGCCAGTAGTATCAACTCAACTGCTGCTAAATGG
501   CAGTCTAGCAGAAGAAGAGGTAGTAATTAGATCTGACAATTTCACGAACA
551   ATGCTAAAACCATAATAGTACAGCTGAAAGAATCTGTAGAAATTAATTGT
601   ACAAGACCCAACAACAATACAAGAAAAAGTATACATATAGGACCAGGGAG
651   AGCATTTTATACTACAGGAGAAATAATAGGAGATATAAGACAAGCACATT
701   GTAACATTAGTAGAGCAAAATGGAATGACACTTTAAAACAGATAGTTATA
751   AAATTAAGAGAACAATTTGAGAATAAAACAATAGTCTTTAATCACTCCTC
801   AGGAGGGGACCCAGAAATTGTAATGCACAGTTTTAATTGTGGAGGAGAAT
851   TTTTCTACTGTAATTCAACACAACTGTTTAATAGTACTTGGAATAATAAT
901   ACTGAAGGGTCAAATAACACTGAAGGAAATACTATCACACTCCCATGCAG
951   AATAAAACAAATTATAAACATGTGGCAGGAAGTAGGAAAAGCAATGTATG
1001  CCCCTCCCATCAGAGGACAAATTAGATGTTCATCAAATATTACAGGGCTG
1051  CTATTAACAAGAGATGGTGGTATTAATGAGAATGGGACCGAGATCTTCAG
1101  ACCTGGAGGAGGAGATATGAGGGACAATTGGAGAAGTGAATTATATAAAT
1151  ATAAAGTAGTAAAAATTGAACCATTAGGAGTAGCACCCACCAAGTGCAAG
1201  AGAAGAGTGGTGCAAAGAGAAAAAAGAGCAGTGGGAATAGGAGCTGTGTT
1251  CCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATGA
1301  CACTGACGGTACAGGCCAGACTATTATTGTCTGGTATAGTGCAACAGCAG
1351  AACAATTTGCTGAGGGCTATTGAGGCGCAACAGCGTATGTTGCAACTCAC
1401  AGTCTGGGGCATCAAGCAGCTCCAGGCAAGAGTCCTGGCTGTGGAAAGAT
1451  ACCTAGGGGATCAACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAACTC
1501  ATTTGCTGCACTGCTGTGCCTTGGAATGCTAGTTGGAGTAATAAATCTCT
1551  GGATAGGATTTGGAATAACATGACCTGGATGGAGTGGGAAAGAGAAATTG
1601  ACAATTACACAAGCGAAATATACACCCTAATTGAAGAATCGCAGAACCAA
1651  CAAGAAAAGAATGAACAAGAATTATTGGAATTAGATAAATGGGCAAGTTT
1701  GTGGAATTGGTTTGACATAACAAACTGGCTGTGGTAT
```

Figure 14

(b)

```
30   VEKLWVTVYY GVPVWKEATT TLFCASDAKA YDTEVHNVWA THACVPTDPN
80   PQEVVLENVT EHFNMWKNNM VEQMQEDIIS LWDQSLKPCV KLTPLCGAGC
130  DTSVITQACP KISFEPIPIH YCAPAGFAIL KCNDKTFNGK GPCKNVSTVQ
180  CTHGIRPVVS TQLLLNGSLA EEEVVIRSDN FTNNAKTIIV QLKESVEINC
230  TRPNNNTRKS IHIGPGRAFY TTGEIIGDIR QAHCNISRAK WNDTLKQIVI
280  KLREQFENKT IVFNHSSGGD PEIVMHSFNC GGEFFYCNST QLFNSTWNNN
330  TEGSNNTEGN TITLPCRIKQ IINMWQEVGK AMYAPPIRGQ IRCSSNITGL
380  LLTRDGGINE NGTEIFRPGG GDMRDNWRSE LYKYKVVKIE PLGVAPTKCK
430  RRVVQREKRA VGIGAVFLGF LGAAGSTMGA ASMTLTVQAR LLLSGIVQQQ
480  NNLLRAIEAQ QRMLQLTVWG IKQLQARVLA VERYLGDQQL LGIWGCSGKL
530  ICCTAVPWNA SWSNKSLDRI WNNMTWMEWE REIDNYTSEI YTLIEESQNQ
580  QEKNEQELLE LDKWASLWNW FDITNWLWY
```

Figure 15

HIV-1_{JR-FL} ΔV3 SOS gp140

(a)

```
1     GTAGAAAAGTTGTGGGTCACAGTCTATTATGGGGTACCTGTGTGGAAAGA
51    AGCAACCACCACTCTATTTTGTGCATCAGATGCTAAAGCATATGATACAG
101   AGGTACATAATGTTTGGGCCACACATGCCTGTGTACCCACAGACCCCAAC
151   CCACAAGAAGTAGTATTGGAAAATGTAACAGAACATTTTAACATGTGGAA
201   AAATAACATGGTAGAACAGATGCAGGAGGATATAATCAGTTTATGGGATC
251   AAAGCCTAAAGCCATGTGTAAAATTAACCCCACTCTGTGTTACTTTAAAT
301   TGCAAGGATGTGAATGCTACTAATACCACTAATGATAGCGAGGGAACGAT
351   GGAGAGAGGAGAAATAAAAAACTGCTCTTTCAATATCACCACAAGCATAA
401   GAGATGAGGTGCAGAAAGAATATGCTCTTTTTTATAAACTTGATGTAGTA
451   CCNATAGATAATAATAATACCAGCTATAGGTTGATAAGTTGTGACACCTC
501   AGTCATTACACAGGCCTGTCCAAAGATATCCTTTGAGCCAATTCCCATAC
551   ATTATTGTGCCCCGGCTGGTTTTGCGATTCTAAAGTGTAATGATAAGACG
601   TTCAATGGAAAAGGNCCATGTAAAAATGTCAGCACAGTNCAATGTACACA
651   TGGAATTAGGCCAGTAGTATCAACTCAACTGCTGCTAAATGGCAGTCTAG
701   CAGAAGAAGAGGTAGTAATTAGATCTGACAATTTCACGAACAATGCTAAA
751   ACCATAATAGTACAGCTGAAAGAATCTGTAGAAATTAATTGTACAAGACC
801   CAACAACAATGGAGCCGGCGATATAAGACAAGCACATTGTAACATTAGTA
851   GAGCAAAATGGAATGACACTTTAAAACAGATAGTTATAAAATTAAGAGAA
901   CAATTTGAGAATAAAACAATAGTCTTTAATCACTCCTCAGGAGGGGACCC
951   AGAAATTGTAATGCACAGTTTTAATTGTGGAGGAGAATTTTTCTACTGTA
1001  ATTCAACACAACTGTTTAATAGTACTTGGAATAATAATACTGAAGGGTCA
1051  AATAACACTGAAGGAAATACTATCACACTCCCATGCAGAATAAAACAAAT
1101  TATAAACATGTGGCAGGAAGTAGGAAAAGCAATGTATGCCCCTCCCATCA
1151  GAGGACAAATTAGATGTTCATCAAATATTACAGGGCTGCTATTAACAAGA
1201  GATGGTGGTATTAATGAGAATGGGACCGAGATCTTCAGACCTGGAGGAGG
1251  AGATATGAGGGACAATTGGAGAAGTGAATTATATAAATATAAAGTAGTAA
1301  AAATTGAACCATTAGGAGTAGCACCCACCAAGTGCAAGAGAAGAGTGGTG
1351  CAAAGAGAAAAAAGAGCAGTGGGAATAGGAGCTGTGTTCCTTGGGTTCTT
1401  GGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATGACACTGACGGTAC
1451  AGGCCAGACTATTATTGTCTGGTATAGTGCAACAGCAGAACAATTTGCTG
1501  AGGGCTATTGAGGCGCAACAGCGTATGTTGCAACTCACAGTCTGGGGCAT
1551  CAAGCAGCTCCAGGCAAGAGTCCTGGCTGTGGAAAGATACCTAGGGGATC
1601  AACAGCTCCTGGGGATTTGGGGTTGCTCTGGAAAACTCATTTGCTGCACT
1651  GCTGTGCCTTGGAATGCTAGTTGGAGTAATAAATCTCTGGATAGGATTTG
1701  GAATAACATGACCTGGATGGAGTGGGAAAGAGAAATTGACAATTACACAA
1751  GCGAAATATACACCCTAATTGAAGAATCGCAGAACCAACAAGAAAAGAAT
1801  GAACAAGAATTATTGGAATTAGATAAATGGGCAAGTTTGTGGAATTGGTT
1851  TGACATAACAAAATGGCTGTGGTAT
```

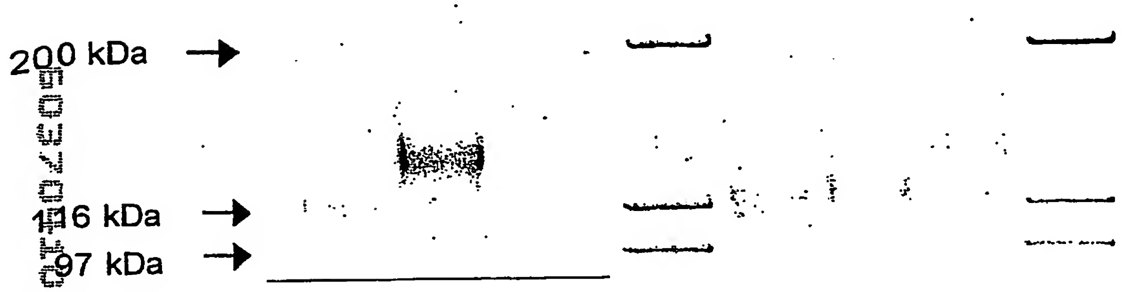
Figure 15

(b)

```
30    VEKLWVTVYY GVPVWKEATT TLFCASDAKA YDTEVHNVWA THACVPTDPN
80    PQEVVLENVT EHFNMWKNNM VEQMQEDIIS LWDQSLKPCV KLTPLCVTLN
130   CKDVNATNTT NDSEGTMERG EIKNCSFNIT TSIRDEVQKE YALFYKLDVV
180   XIDNNNTSYR LISCDTSVIT QACPKISFEP IPIHYCAPAG FAILKCNDKT
230   FNGKXPCKNV STXQCTHGIR PVVSTQLLLN GSLAEEEVVI RSDNFTNNAK
280   TIIVQLKESV EINCTRPNNN GAGDIRQAHC NISRAKWNDT LKQIVIKLRE
330   QFENKTIVFN HSSGGDPEIV MHSFNCGGEF FYCNSTQLFN STWNNNTEGS
380   NNTEGNTITL PCRIKQIINM WQEVGKAMYA PPIRGQIRCS SNITGLLLTR
430   DGGINENGTE IFRPGGGDMR DNWRSELYKY KVVKIEPLGV APTKCKRRVV
480   QREKRAVGIG AVFLGFLGAA GSTMGAASMT LTVQARLLLS GIVQQQNNLL
530   RAIEAQQRML QLTVWGIKQL QARVLAVERY LGDQQLLGIW GCSGKLICCT
580   AVPWNASWSN KSLDRIWNNM TWMEWEREID NYTSEIYTLI EESQNQQEKN
630   EQELLELDKW ASLWNWFDIT KWLWY
```

Figure 16

reduced				non-reduced				
1	2	3	4	5	6	7	8	9
MW STD	gp120	SOS gp140	gp140 UNC	MW STD	none	gp120	SOS gp140	gp140 UNC



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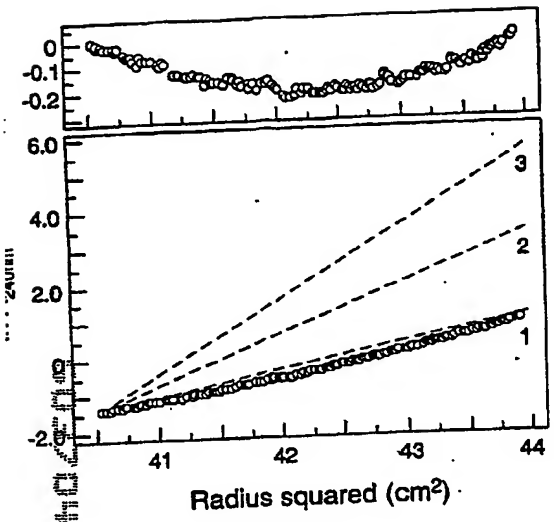

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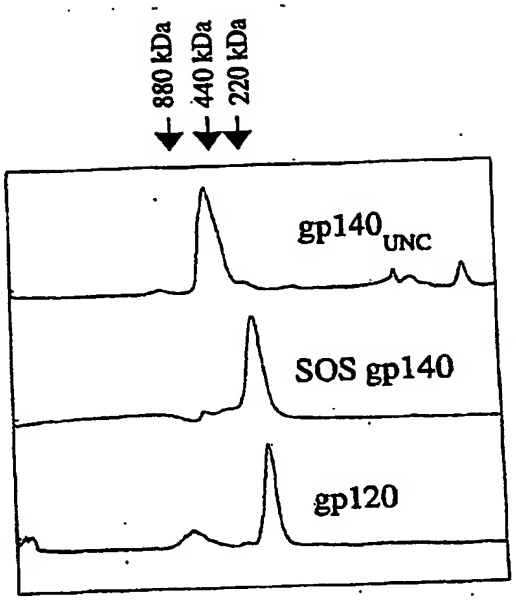

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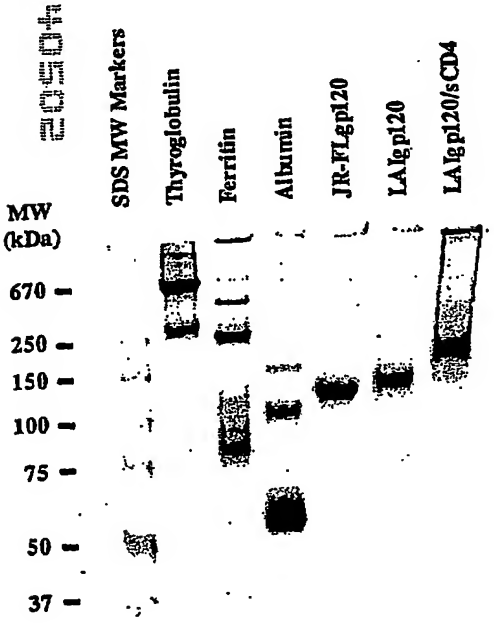

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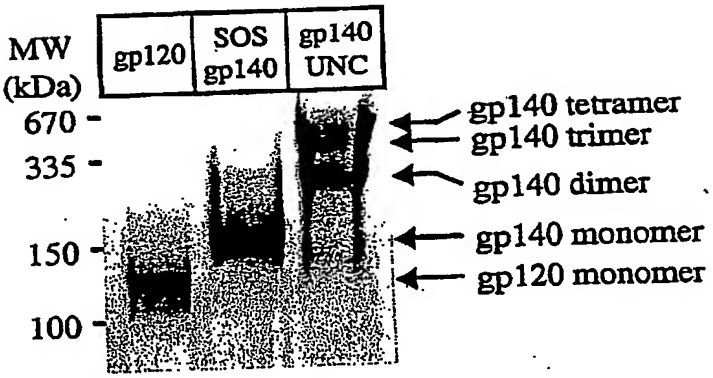

Figure 18

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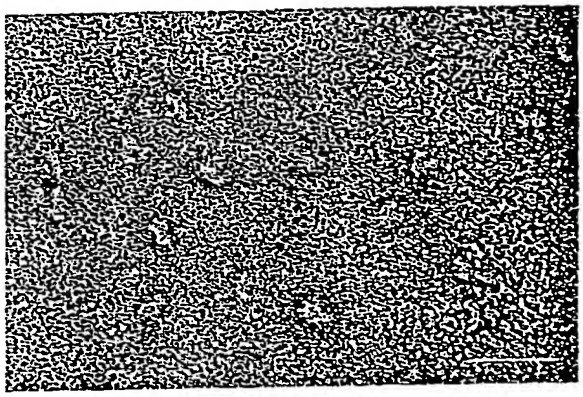

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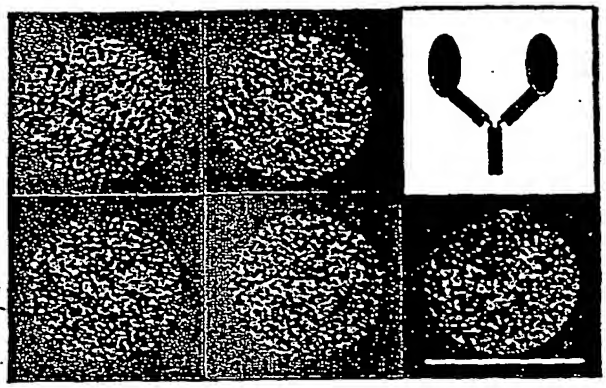

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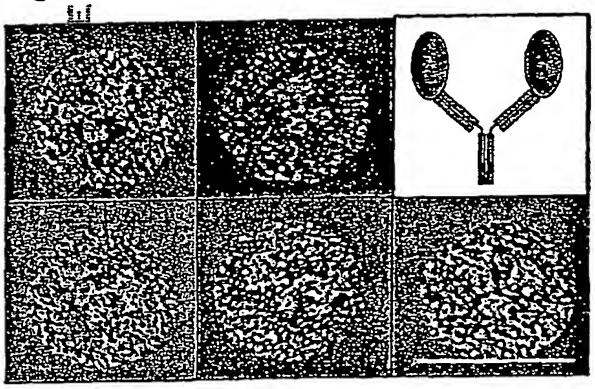

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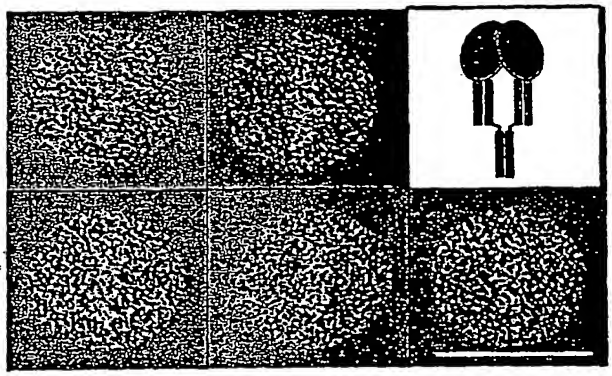

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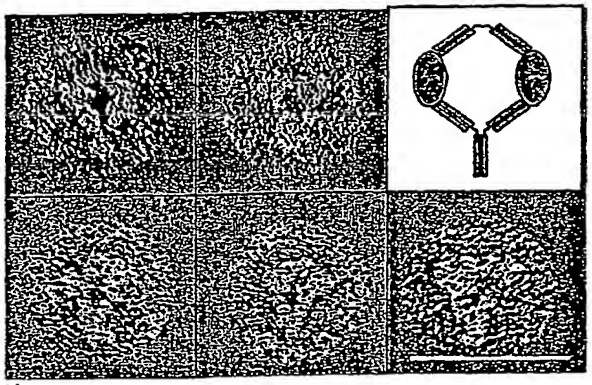

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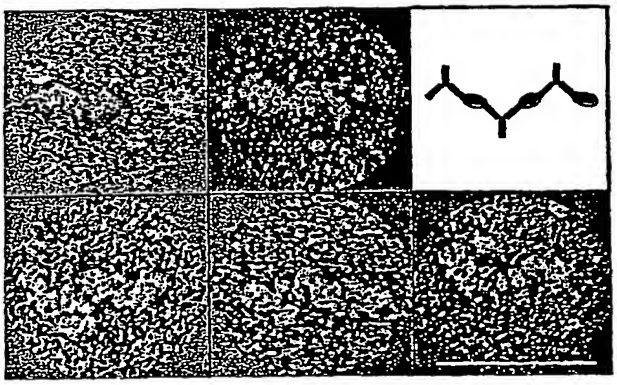

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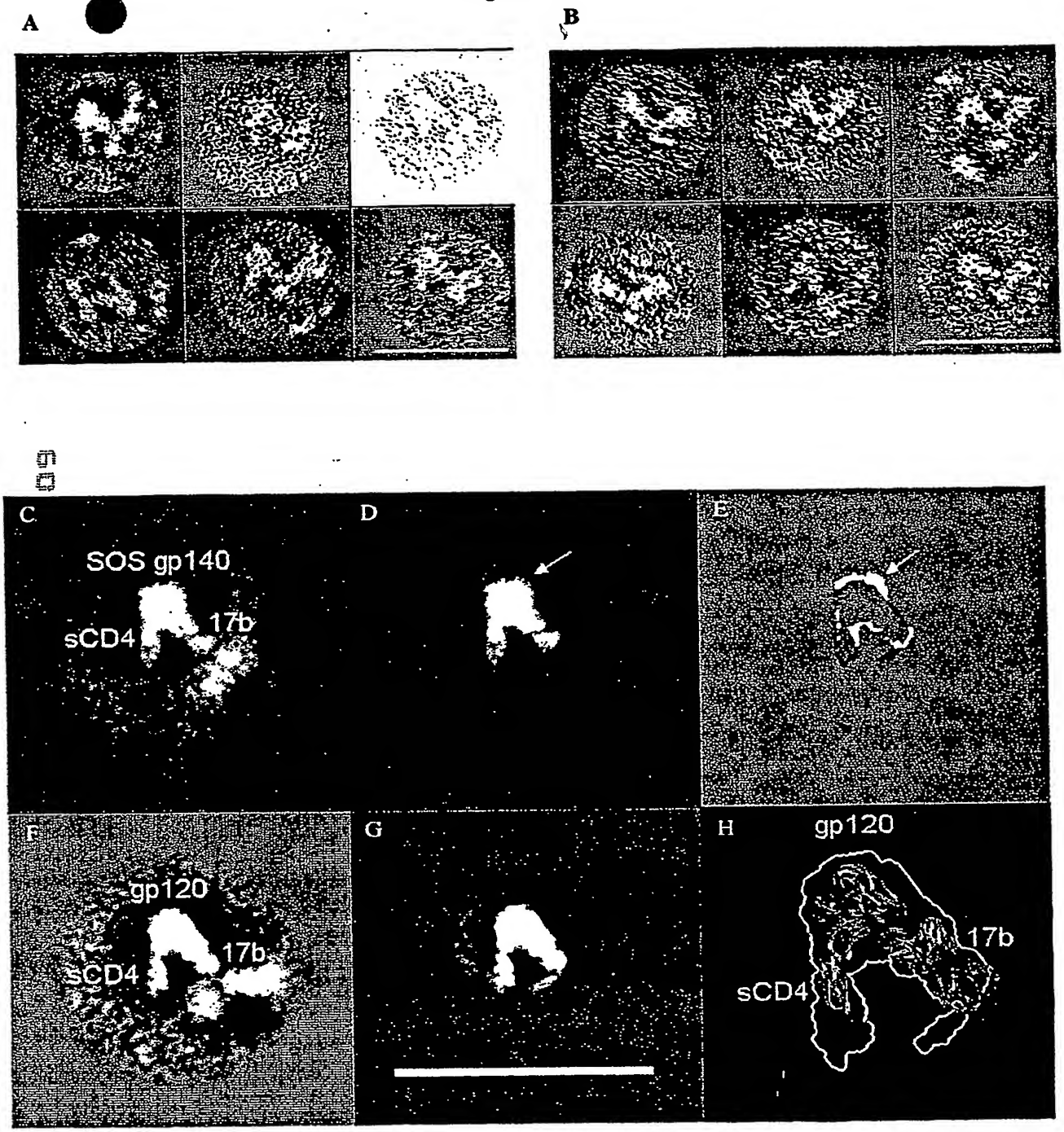

Figure 20

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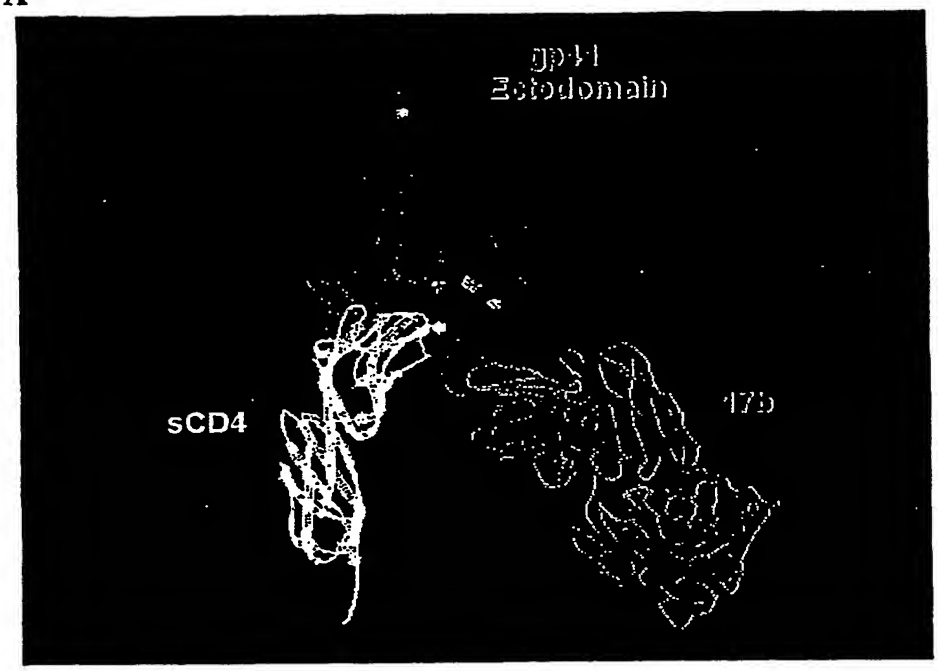
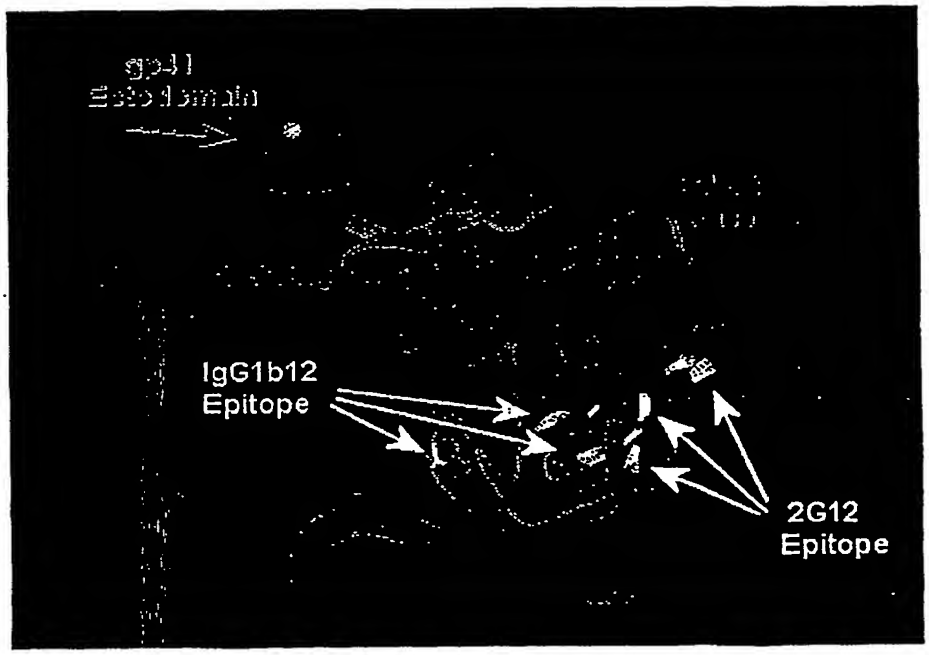

gp41
Ectodomain
sCD4
17b

B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Ectodomain
IgG1b12
Epitope
2G12
Epitope

Figure 21

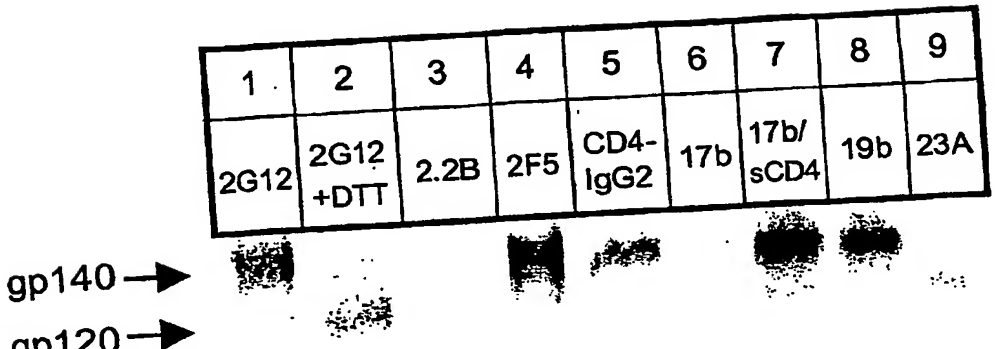
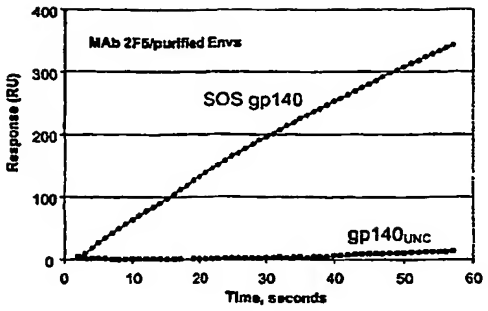

1
2
3
4
5
6
7
8
9
2G12
2G12 +DTT
2.2B
2F5
CD4-IgG2
17b
17b/ sCD4
19b
23A
gp140
gp120

Figure 22

A



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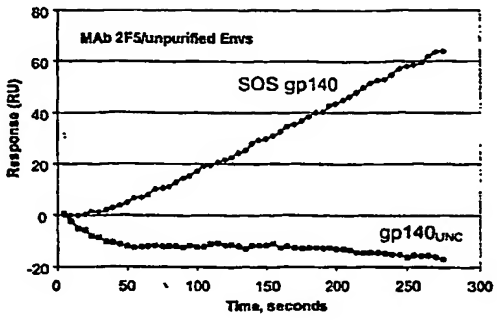

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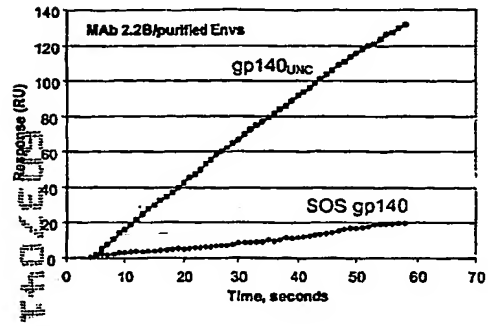

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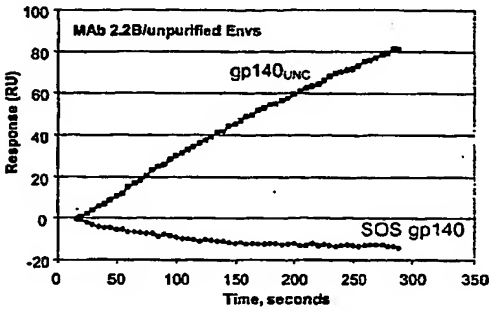

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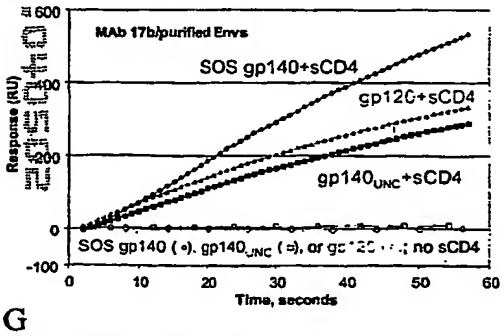

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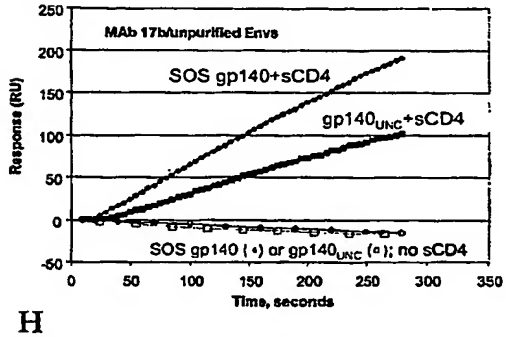

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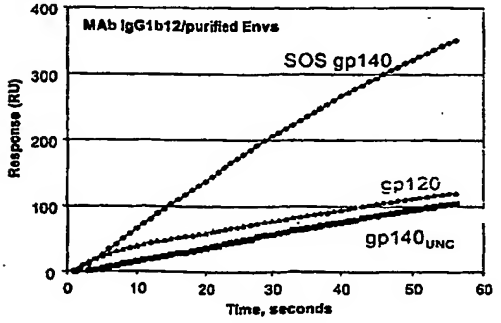

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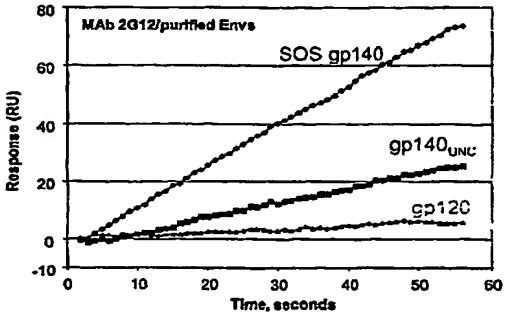

Figure 23

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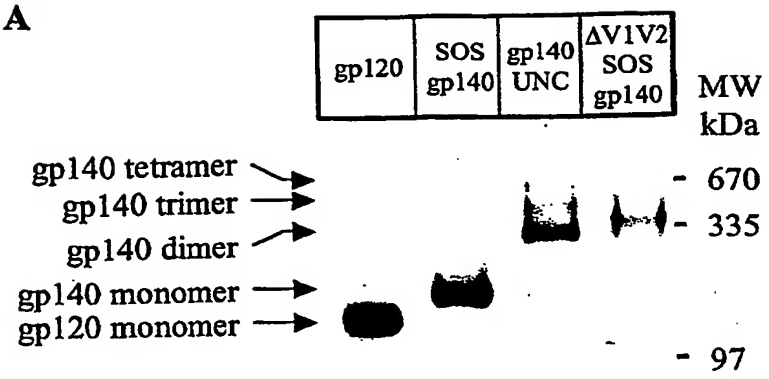

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